

1987

# Biotechnology applications of Populus micropropagation

Young Woo Chun  
*Iowa State University*

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**Biotechnology applications of *Populus* micropropagation**

**Chun, Young Woo, Ph.D.**

**Iowa State University, 1987**

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Biotechnology applications of *Populus* micropropagation

by

Young Woo Chun

A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of the  
Requirements for the Degree of  
DOCTOR OF PHILOSOPHY

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Iowa State University  
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1987



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## GENERAL INTRODUCTION

## Explanation of Dissertation Format

This dissertation is presented in the alternative format style that is outlined in the Graduate College Thesis Manual Handbook, Iowa State University, Ames, IA (Revised ed., 1987). The dissertation consists of a general introduction followed by individual papers that have been prepared for submission to scholarly journals. These are followed by an overall summary and discussion section that serves to unite the individual papers. The papers included in the dissertation consist of six parts. The first, influences of medium consistency and shoot density on *in vitro* shoot proliferation, was the result of an independent project in a plant tissue culture course (Hort. 523 taught by Dr. Stephens). One of the collaborators (Dr. Hall) provided the initial idea of liquid culture on *in vitro* shoot proliferation of *Populus*. The second part represents a technique for protoplast isolation and culture from *in vitro* cultured *Populus* explants. The expenses for this part were provided by the Graduate College. The third part deals with the morphogenetic potential of *in vitro* cultured *Populus* explants. This work became necessary to provide a vehicle for transformation studies of *Populus* using *Agrobacterium*. Technical support and expenses were provided by Drs. Klopfenstein and Hall. Statistical advice was provided by the other junior author, Dr. Mize. The fourth and fifth parts comprise cold storage studies of *in vitro* cultured plantlets, and polyploidy induction of *Populus*. Dr. Hall provided expenses and technical support under the

project on North Central Regional Project NC-99, Regional Tree Improvement (Iowa Agriculture and Home Economics Experiment Station). The last section concerns transformation of *Populus* species. For this study, I and Dr. Klopfenstein designed *Agrobacterium* host range and kanamycin concentration tests. Drs. McNabb and Hall provided materials and technical support. Supplementary expenses were also provided the Graduate College.

### Biotechnological Applications in *Populus* Species

#### Introduction

It was first reported in 1983 that a plant gene was transferred to and expressed in a plant of a different species using an *Agrobacterium*-mediated gene transfer method (Hoekema et al., 1983; Murai et al., 1983). Since then, gene transfer methods have been developed for several important forest tree species such as *Populus alba* X *P. grandidentata* (Fillatti et al., 1987), *Pinus taeda* (Sederoff et al., 1986) and *Pseudotsuga menziesii* (Dandekar et al., 1987). In addition, major applications of tissue culture techniques for forest tree improvement were utilized for *Populus* species (Fröhlich and Weisgerber, 1985), *Pinus taeda* (McKeand and Weir, 1984), and *Pinus radiata* (Aitken-Christie and Gleed, 1984). Because of rapid progress in developing biotechnological applications with *Populus* species, this genus is well-suited as a model system for deciduous forest tree species.

In this paper, the development of tissue culture systems for *Populus* species, and the utilization of tissue culture biotechnology will be

reviewed and discussed. Special emphasis will be placed on prospects for genetic transformation by *Agrobacterium*-mediated gene transfer methods.

### Forest biotechnology

There are several different definitions of biotechnology.

Biotechnology can be defined broadly as a technology that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop micro-organisms for specific uses (Sederoff and Ledig, 1984). Torrey (1985) defined plant biotechnology as "the application of existing techniques of plant organ, tissue, and cell culture, plant molecular biology, and genetic engineering to the improvement of plants and of plant productivity for the benefit of man". Recently, Nelson and Haissig (1984) defined forest biotechnology as micropropagation and pollen suppression as well as genetic engineering including DNA recombination and protoplast fusion. Gupta and Durzan (1987) and Haissig et al. (1987) also used the biotechnology term in the area of tissue culture as tissue culture biotechnology. Chun (1985b) defined forest biotechnology as shown in Figure 1. For other discussions of the potential applications of biotechnology to forest tree species, the reader is referred to other reviews by Sommers and Brown (1979), Karnosky (1981), Farnum et al. (1983), Faltonson et al. (1984), Sederoff and Ledig (1984), Chun (1985a), Ahuja (1986) and Haissig et al. (1987).

One of the most valuable features of forest biotechnology is that it allows rapid incorporation of specific improvements within a short period of time. Nelson and Haissig (1984) and Riemenschneider et al. (1987) have shown that biotechnology can reduce the long time periods required for tree improvement by conventional tree breeding technology alone.

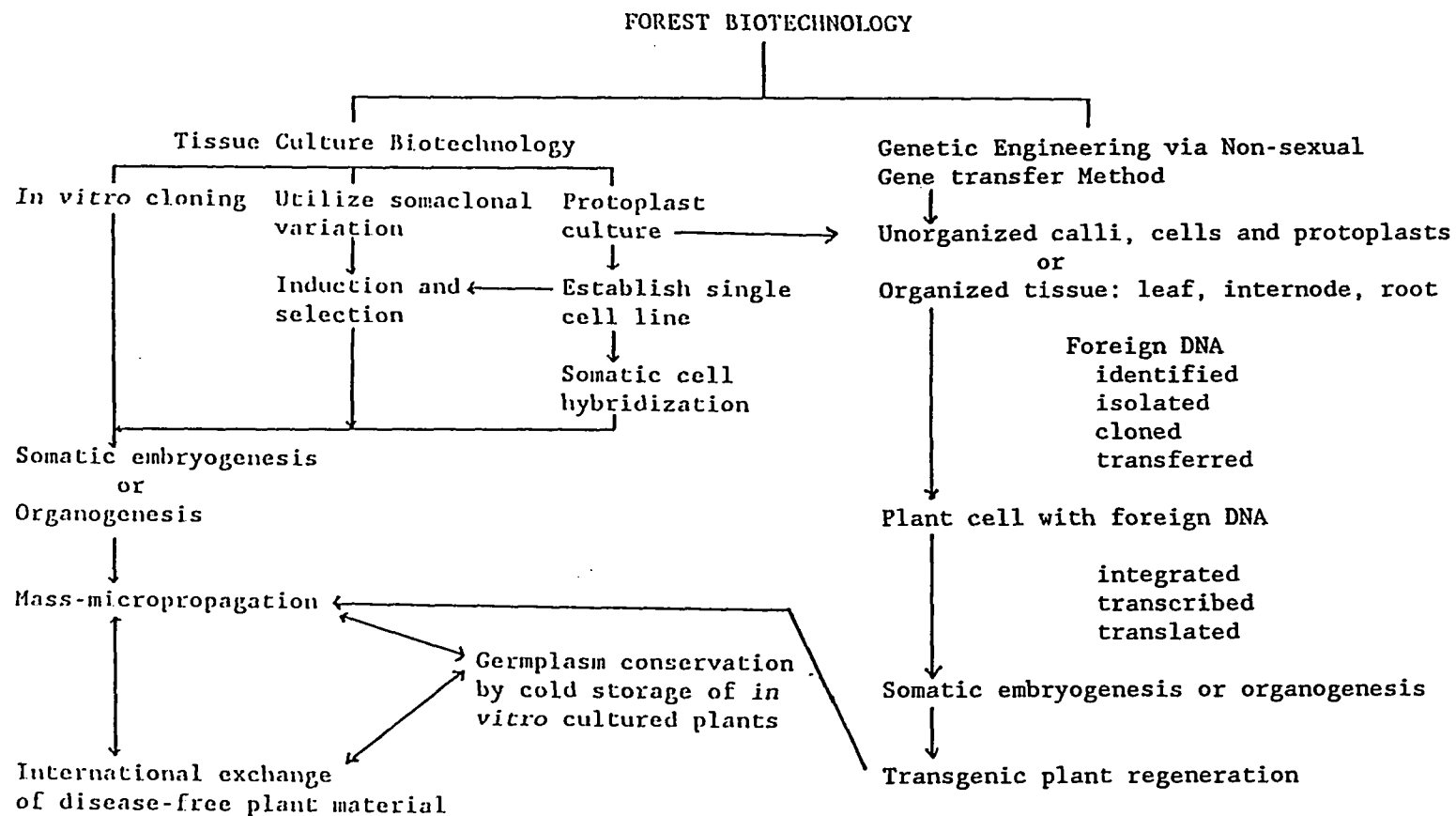


Figure 1. Forest biotechnology by tissue culture and genetic engineering. On the left, established and developing *in vitro* techniques; on the right, prospective genetic engineering developments.

Populus as a model system for biotechnological applications with deciduous forest tree species

There is a firm basis to support the use of *Populus* species as a model system for biotechnological applications with deciduous forest tree species. Most biotechnological applications with plants hinge on the ability to manipulate the morphogenetic potential of cells and tissue in culture.

Among the forest tree species, *Populus* species are the most well-studied in the area of tissue culture (Table 1). Tissue of *Populus* exhibits a high degree of developmental plasticity, similar to that of tobacco in the herbaceous species. *Populus* species are the only forest tree species for which *in vitro* systems have been defined to date for regeneration from protoplasts of leaf mesophyll tissue, sporophytic calli, gametophytic (anther) calli, leaf discs, internodes, root segments, axillary buds, and apical meristems. *Populus* species are the only woody species that have been transformed with a silviculturally-useful gene by an *Agrobacterium*-mediated gene transfer method (Fillatti et al., 1987). There is also one report in which embryo rescue techniques were utilized to circumvent incompatibility of pollination between two different *Populus* species (Noh et al., 1986).

In relation to other aspects of molecular biology, *Populus* species have a relatively small genome size which greatly facilitates the production and screening of genomic libraries (Parsons et al., 1986) (Table 2). *Populus* species also are promising candidates as recipients for Ti plasmid-mediated gene transfer. Earlier work reports the susceptibility of various *Populus* species to infection by naturally-



Table 1. Tissue culture research with *Populus* species

Year	Species	Culture <sup>a</sup>	Regen. <sup>b</sup>	Reference
1964	<i>P. tremuloides</i>	C	S R	Mathes a,b
1968	<i>P. tremuloides</i>	C	S R W	Winton a,b
	<i>P. tremuloides</i>	C	S R	Wolter
1970	<i>P. tremuloides</i>	C	S R W	Winton
1971	<i>P. tremula</i>	C	S R W	Winton
1972	<i>P. deltoides</i> X <i>P. nigra</i>	C	S R W	Berbee et al.
	<i>P. trichocarpa</i>	Cat	S	Bawa and Stettin
1973	<i>P. nigra</i> 'Italica'	C	S R	Venverloo
1974	<i>P. euramericana</i>	C	S R	Chalupa
	<i>P. tremula</i>	C	S R	Chalupa
	<i>P. deltoides</i>	A	S R	Sato
	<i>P. Sieboldii</i> X <i>P. grandidentata</i>	A	S R	Sato
	<i>P. maximowiczii</i>	A	S R	Sato
1975	<i>P. nigra</i>	A	S R	Wang et al.
	<i>P. tremuloides</i>	C		Wolter and Gordon
	<i>P. ussuriensis</i>	A	S R	Heilungkiang Inst.
	<i>P. simonii</i> X <i>P. nigra</i>	A	S R	Heilungkiang Inst.

<sup>a</sup>Culture - type of tissue culture. C: callus culture; S: shoot tip (axillary bud) culture; Cat: catkin culture; AM: apical meristem culture; A: anther culture; I: internode culture; IC: immature embryo culture; EP: cultured explant culture; CS: *in vitro* cold storage; ER: immature embryo rescue; T: transformation; SV: induction and selection of somaclonal variation; IE: *in vitro* culture for international exchange.

<sup>b</sup>Regen. - S: shoot regeneration; R: root regeneration; W: whole plant regeneration.

Table 1. Continued

Year	Species	Culture	Regen	Reference
1977	<i>P. euramericana</i>	C	S R	Lester and Berbee
	<i>P. tristis</i> X			
	<i>P. balsamifera</i>	A.M	S R W	Thompson and Gordon
	<i>P. nigra</i> 'Italica'	S	S R W	Whitehead and Giles
	<i>P. deltoides</i> X <i>P. nigra</i>	S	S R W	Whitehead and Giles
	<i>P. yunnanensis</i>	S	S R W	Whitehead and Giles
	<i>P. berolinensis</i>	A	S R W	North East For. Acad.
	<i>P. harbinensis</i> X			
	<i>P. pyramidalis</i>	A	S R W	North East For. Acad.
	<i>P. canadensis</i> X			
	<i>P. koreana</i>	A	S R W	North East For. Acad.
1978	<i>P. alba</i> ; <i>P. canescens</i>	S	S R W	Christie
	<i>P. tremula</i> ; <i>P. alba</i>	S	S R W	Christie
	<i>P. alba</i> X <i>P. tremula</i>	S	S R W	Christie
	<i>P. tremuloides</i>	S	S R W	Christie
	<i>P. alba</i> X <i>P. glandulosa</i>	S	S R W	Christie
1980	<i>P.</i> 14 species	A	S R W	Zhu et al.
	<i>P. euramericana</i> I-214	C	S	Saito a
	<i>P. euramericana</i> I-45/51	C	S	Saito b
	<i>P. deltoides</i>	C	S	Saito b
1981	<i>P. Sieboldii</i> X			
	<i>P. grandidentata</i>	C	S R	Saito and Kawanobe
	<i>P. alba</i> X <i>P. glandulosa</i>	C	S R W	Kim et al.
1982	<i>P. maximowiczii</i> X			
	<i>P. deltoides</i>	A	S R	Ho
1983	<i>P. tremula</i>	S	S R W	Ahuja a
	<i>P. tremuloides</i>	S	S R W	Ahuja a
	<i>P. glandulosa</i>	A	S R W	Kim et al.
1984	<i>P. alba</i> X			
	<i>P. grandidentata</i>	S	S R W	Chun and Hall
	<i>P. trichocarpa</i> X			
	<i>P. tacamahaca</i>	I	S R W	Douglas
	<i>P. deltoides</i>	IC	S R	Kouider et al.
1985	<i>P. heterophylla</i>	IC	S R	Savka and Dawson

Table 1. Continued

Year	Species	Culture	Regen	Reference
1986	<i>P. alba</i> X			
	<i>P. grandidentata</i>	S, CS	S R W	Chun and Hall
	<i>P. tremuloides</i>	C	S R W	Noh and Minocha
	<i>P. tremuloides</i>	EP	S R W	Wann and Einspahr
	<i>P. maximowiczii</i>	A	S R W	Kim et al.
	<i>P. alba</i> X <i>P. glandulosa</i>	C	S R W	Kim et al.
	<i>P. davidiana</i> X			
	<i>P. deltoides</i>	ER	S R W	Noh et al.
	<i>P. maximowiczii</i> X			
	<i>P. glandulosa</i>	ER	S R W	Noh et al.
	<i>P. nigra</i> X <i>P. trichocarpa</i>	SV	S R W	Ettinger et al.
	<i>P. trichocarpa</i>	IE	S R W	McNabb et al.
	<i>P. deltoides</i> X			
	<i>P. trichocarpa</i>	IE	S R W	McNabb et al.
	<i>P. nigra</i> X			
	<i>P. trichocarpa</i>	IE	S R W	McNabb et al.
	<i>P. deltoides</i> X			
	<i>P. maximowiczii</i>	IE	S R W	McNabb et al.
1987	<i>P. nigra</i> X <i>P. trichocarpa</i>	SV	S R W	Ostry and Skilling
	<i>P. maximowiczii</i> X			
	<i>P. trichocarpa</i>	SV	S R W	Michler and Bauer
	<i>P. alba</i> X			
	<i>P. grandidentata</i>	T	S R W	Fillatti et al.
	<i>P. alba</i> X			
	<i>P. grandidentata</i>	EP	S R W	Chun

occurring *Agrobacterium tumefaciens* (De Cleene and Deley, 1976).

Phytohormone independent callus growth has been obtained from stem and shoot segments of *Populus trichocarpa* x *P. deltoides* (*P. X interamericana* Brockh.) following transformation by wild type *Agrobacterium tumefaciens* strains A281 and A348 (Parsons et al., 1986).

Table 2. Range of 1C nuclear DNA content per cell values for selected plants

species	DNA content (picogram)	Reference
Soybean	1.2	Grierson and Covey, 1984
Tobacco	2.0	Grierson and Covey, 1984
<i>Populus deltoides</i>	0.535	Dhillon, 1987
<i>Pinus taeda</i>	12.0	Sederoff et al., 1986
<i>Pinus lambertiana</i>	43.8	Rake et al., 1980
<i>Picea glauca</i>	9.65	Rake et al., 1980
<i>Pinus banksiana</i>	14.9	Rake et al., 1980
<i>Pinus resinosa</i>	21.55	Rake et al., 1980
<i>Pinus strobus</i>	19.4	Kriebel, 1985
<i>Pinus caribaea</i>	12.5	Berlyn et al., 1987

With regard to silvicultural aspects, *Populus* species are among the fastest growing deciduous forest tree species and are distributed throughout temperate forests of the northern hemisphere (Dickmann and Stuart, 1983). Because of their rapid growth, ease of establishment through stem or root cuttings, apparent ease of coppice regeneration, *Populus* species have been considered ideal species for pulpwood and lumber production (Schreiner, 1974; Hall et al., 1982). In addition, species and hybrids of *Populus* have been studied extensively for use in short-rotation, intensively cultured plantation systems for woody biomass production (Hall, 1985). Trees of this genus also represent wide genetic

diversity, exhibit a wide range of site requirements, and respond well to cultural inputs (Schreiner, 1974).

#### Tissue culture biotechnology of *Populus*

Micropropagation Tissue culture systems for woody plant species have been developed primarily during the past two decades (Hartmann and Kester, 1983). For a diverse woody species, the application of tissue culture techniques for mass-propagation has become a primary alternative to more conventional propagation procedures. Economic factors currently restrict the large-scale utilization of tissue culture for mass propagation of poplar species (Haissig et al., 1987); however, two routine methods, axillary bud production (also termed axillary shoot production) and adventitious shoot initiation, have been developed for experimental use with these woody species.

Several *Populus* species have been successfully micropropagated in tissue culture systems using axillary bud culture and adventitious shoot initiation from cultured explants. Whitehead and Giles (1977) and Christie (1978) have shown that *Populus* species can be mass-propagated by axillary bud culture. There are also several reports that demonstrate the versatility of *in vitro* cultured poplar plantlets as a source material for biotechnological applications in manners such as: 1) direct morphogenesis from culture explants (Wann and Einspahr, 1986; Chun, 1987), 2) induction and selection of somaclonal variation for disease resistance (Ettinger et al., 1986; Ostry and Skilling, 1987) or herbicide tolerance (Nelson and Haissig, 1986; Michler and Bauer, 1987), 3) protoplast isolation and culture (Chun, 1985b; Russell and McCown, 1986), 4) ploidy level

manipulation (Chun, 1987), 5) international exchange of disease-free plant materials (McNabb et al., 1986), 6) germplasm preservation through cold storage (Chun and Hall, 1986; Chun, 1987), and 7) regeneration of shoots transformed via co-culture with a genetically engineered *Agrobacterium* Ti plasmid binary vector system (Fillatti et al., 1987). The advantages of using *in vitro* bud-cultured plantlets as a source material for biotechnological application are based on the following: 1) *in vitro* bud culture provides stable genotype sources (Lawrence, 1981; Karp and Bright, 1985), 2) a juvenile growth condition can be maintained under *in vitro* conditions (Scorza and Janick, 1980), 3) it provides a high morphogenetic potential for organogenesis (Wann and Einspahr, 1986; Chun, 1987), and 4) it also can provide a year-round plant source with a cyclical growth phase, which can be contained within a small space.

Somaclonal variation      The technology of introducing and (or) selecting genetic variation by using tissue culture has been termed somaclonal variation (Larkin and Scowcroft, 1981; Evans et al., 1984). Somaclonal variation has been observed for several phenotypic characteristics (Skirvin, 1978; Karp and Bright, 1985). The phenotypic characteristics of somaclonal variation can be caused by a range of genotypic or epigenetic changes. One of the most promising features of somaclonal variation is that it can involve agronomically-useful traits such as yield or disease resistance. The potential of somaclonal variation for improvement for woody species and crops has been reviewed by several scientists (Larkin and Scowcroft, 1981; Evans, 1986; Haissig et al., 1987). Somaclonal variation has been utilized with a range of plant species including cereals (maize, oats, wheat, rice), seed crops (tobacco,

tomato, alfalfa), vegetatively propagated crops (sugarcane, potato) and woody species (poplar) (Lester and Berbee, 1977). It is evident that the tools of somaclonal variation are technologies that can permit short-term accomplishment of breeding objectives (Haissig et al., 1987). Somaclonal variation cannot replace conventional plant breeding, but it can generate or select desired variation to facilitate conventional breeding processes (Haissig et al., 1987).

With regard to *Populus* species, Lester and Berbee (1977) demonstrated that callus culture-derived hybrid poplar (*P. deltoides* X *P. nigra*) exhibited somaclonal variation in height, number of branches, and leaf traits after one growing season. There are other reports in which somaclonal variation was utilized to select septoria leafspot-canker tolerant, hybrid poplar (Ettinger et al., 1986; Ostry et al., 1986; Ostry and Skilling, 1987) and herbicide tolerant, hybrid poplar (Nelson and Haissig, 1986; Michler and Bauer, 1987). Haissig et al. (1987) predict that applications of somaclonal and gametoclonal variation in tissue culture biotechnology of woody species will be the primary strategies for specific tree improvements of a monogenic or oligogenic nature.

Protoplast culture One of the most significant developments in tissue culture techniques within recent years has been the isolation and culture of protoplasts. Such techniques have several potential applications, including: 1) selection of useful somaclonal variation in plants regenerated from protoplasts (Shepard et al., 1980; Kemble and Shepard, 1984), 2) insertion of silviculturally-important genes by micro-injection, electroporation, or *Agrobacterium* Ti plasmid-derived system (Ahuja, 1985; Cocking and Davey, 1987), 3) producing somatic cellular

hybridization (Gleba and Sytnik, 1984), 4) uptake of nuclei, organelles, chromosomes or macro-molecules such as DNA and RNA (Saxena et al., 1986), and 5) physiological studies (Vasil, 1976). In recent years, protoplasts have been routinely isolated, cultured, and regenerated with a number of plant species (Ahuja, 1982; 1984). Most woody plants, however, remain among the recalcitrant species that are difficult to regenerate from the protoplast cultures. Nevertheless, successful protoplast isolations or cultures have been regenerated with a few species of *Populus* (Table 3). Recently, Russell and McCown (1986) regenerated whole plants from single protoplasts of *Populus alba* X *P. grandidentata*. Since protoplasts from forest tree species remain difficult to grow and differentiate *in vitro*, it has not yet been possible to effectively utilize somatic hybridization techniques to produce new genotypes.

Table 3. Protoplast research in *Populus* species

Species	Reference
<i>P. euramericana</i> cv. I-45/51	Saito, 1976, 1980c
<i>P. trichocarpa</i> X <i>P. tacamahaca</i>	Douglas, 1982
<i>P. tremuloides</i>	Verma and Wann, 1983
	Ahuja, 1983b
<i>P. alba</i> X <i>P. grandidentata</i>	Chun, 1985b
	Russell and McCown, 1986
<i>P. tremula</i> X <i>P. tremuloides</i>	Ahuja, 1984
<i>P. tremula</i>	Ahuja, 1984
<i>P. nigra</i> X <i>P. laurifolia</i>	Russell and McCown, 1986
<i>P. tacatricho</i>	Butt, 1985
<i>P. euramericana</i> I-214	Park and Son, 1986
<i>P. alba</i> X <i>P. glandulosa</i>	Park and Han, 1986

#### Limitation of tissue culture biotechnology in *Populus* species

*Populus* species are promising candidates as the target species for tissue



culture biotechnology applications because recent work has demonstrated rapid progress in specific areas such as micropropagation, protoplast culture, and induction of beneficial somaclonal variation. This recent progress should stimulate more rapid incorporation of specific improvements with conventional tree breeding programs within a short period of time. There are still many basic problems, however, to overcome for utilization of biotechnology in *Populus* species. These problems include the limitations in 1) somatic embryogenesis, 2) generalized regeneration technique for protoplast culture, 3) uncontrolled somaclonal variation leading to a failure of true-to-type propagation in tissue culture.

#### Genetic engineering of *Populus*

To utilize genetic engineering in *Populus* species, there are four general considerations: 1) What genes and regulatory regions are available for transfer into target species? 2) What delivery systems can be used to transfer the specific gene? 3) Are systems established for regeneration from tissue culture? and 4) Are selection methods available to screen for transformed tissue or whole plants?

Possible genes for transformation Recently, several research groups have isolated and transferred a few agriculturally-useful genes into plants. These include genes for insect resistance such as proteinase inhibitor II gene from potato (Thornburg et al., 1987a; b), trypsin inhibitor gene from cowpea (Klausner, 1987), and insecticidal protein genes from *Bacillus thuringiensis* (Adang et al., 1986; Fischhoff et al., 1987), disease resistance such as coat protein genes from tobacco mosaic virus (Abel et al., 1986) and alfalfa mosaic virus (Tumer et al., 1987),

and herbicide tolerance from bacteria (Comai et al., 1984) or from a plant (Shah et al., 1986). Sederoff and Ledig (1984) reported some possible gene systems with potential for tree improvement. These single dominant genes include a blister rust resistance gene in white pine, an apical dominance gene in Scots pine, and a cedar leaf blight-resistance gene in Western red cedar and Japanese red cedar. Like other tree species, *Populus* species have the same difficulties in identifying and manipulating genes responsible for expression of traits that are under single- or oligo-genic control. So far, the promoter region of proteinase inhibitor gene is the only identified one in *Populus* (Parsons at Department of Biochemistry, Univ. of Washington: personal communication). In addition, a bacterial 5-enolpyruvyl-shikimate 3-phosphate (EPSP) synthase gene was used to transform poplar and expressed to provide glyphosate tolerance (Fillatti et al., 1987).

Gene transfer method      There presently are several non-sexual gene transfer methods available for introducing DNA sequences into plant cells (Goodman et al., 1987; Perani et al., 1986; Wilke-Douglas et al., 1986; Konez et al., 1987). These methods include: 1) protoplast (cell) fusion, 2) gene transfer by manipulating DNA directly, 3) *Agrobacterium*-mediated gene transfer, 4) microinjection, 5) virus-mediated gene transfer, and 6) transposon-mediated gene transfer. Among these different gene transfer methods, *Agrobacterium*-mediated gene transfer method is, apparently, the only method reportedly used thus far for woody species (Fillatti et al., 1987). Direct DNA uptake methods of plant gene transfer require plant cell protoplasts, which are difficult to regenerate for most woody species. This barrier can be circumvented with an *Agrobacterium*-mediated

gene transfer system, because transformed plant material can be produced and regenerated with this gene transfer method without protoplast isolation and culture. As a result, such tissue culture-related limitations of woody species are diminished with *Agrobacterium*-mediated gene transfer methods.

*Agrobacterium*-mediated gene transformation      *Agrobacterium*-mediated gene transfer utilizes the natural ability of *Agrobacterium tumefaciens* to infect many dicotyledonous and gymnospermous plants. *Agrobacterium* infection causes tumorous crown gall formation by introducing DNA into the plant cells at a wound site. Major events in the development of *Agrobacterium*-mediated gene transfer methods are summarized in Table 4. The recent discovery that the T-DNA and the *vir* region of *Agrobacterium tumefaciens* Ti plasmid could be separated into two different plasmids, without loss of the transfer capability of the T-DNA, promoted the construction of *Agrobacterium* binary vector systems. These binary vector plant transformation systems consist of two plasmids in *Agrobacterium*: the binary vector plasmid and a helper plasmid (Hoekema et al., 1983; An et al., 1985). A typical binary T-DNA plasmid vector contains several useful characteristics such as 1) T-DNA border sequences which are required for successful transfer of DNA from the *Agrobacterium* cell into the plant genome, 2) A wide host range replicon which can replicate in both *Escherichia coli* and *Agrobacterium* cell systems. The desired foreign gene is cloned into the engineered binary T-DNA plasmid between the border sequences, 3) A selectable marker gene that usually confers antibiotic resistance to allow selection of transformed plant material. The typical helper Ti plasmid is an intact wild type or a disarmed (T-DNA deleted) Ti

Table 4. Major events toward developing *Agrobacterium*-mediated gene transfer methods

Year	Major events
1974	-Identification of Ti plasmid (Zaenen et al.)
1977	-Stable transformation of T-DNA into higher plant cells (Chilton et al.)
1980	-T-DNA integration into plant nuclear genome by insertion of a foreign gene into the T-DNA region of a Ti plasmid (Chilton et al.; Willmitzer et al.)
1981	-Functional map of T-DNA delineated (Garfinkel et al.)
1982	-Determination that virulence ( <i>vir</i> ) genes of Ti plasmid are required for T-DNA integration into plant nuclear genome (Iyer et al.)
1983	-Selective marker genes conferring resistance to antibiotics were inserted into a Ti plasmid for selection of transgenic plant tissue (Herrera-Estrella et al., a; b) -Development of transformation techniques by homologous recombination of hybrid and wild type Ti plasmid in <i>Agrobacterium tumefaciens</i> (Barton et al.; Zambryski et al.) -First plant gene (bean phaseolin gene) expressed in a plant of a different species (sunflower) by Ti plasmid (Murai et al.) -Development of binary T-DNA vectors for the transfer of a foreign gene (pea small subunit gene of ribulose 1,5-bisphosphate carboxylase) into a plant (Hoekema et al.)
1984	-Determined that T-DNA border sequences (right and left borders) are essential for transformation (Wang et al.; Shaw et al.) -Obtained expression of foreign genes in regenerated plants and their progeny (Herrera-Estrella et al.)
1985	-Utilized a binary vector system of <i>Agrobacterium</i> for transformation of leaf discs (An et al.; Klee et al.)
1986	-Transformation of hybrid poplar and loblolly pine ( <i>Pinus taeda</i> ) by Ti plasmid (Parsons et al.; Sederoff et al.)
1987	-Regeneration of glyphosate-tolerant hybrid poplar produced by <i>Agrobacterium</i> binary vector system (Fillatti et al.) -A kanamycin resistance marker gene was transferred to Douglas-fir by <i>Agrobacterium</i> (Dandekar et al.)

plasmid that usually contain supervirulent (broad host range) *vir* genes.

The *vir* genes on a helper Ti plasmid can act in *cis* to promote transfer of the T-DNA of its own plasmid or in *trans*, to transfer the T-DNA on the binary plasmid (Hille et al., 1984).

Selectable markers for plant transformation Several marker genes for plant transformation have been developed (Fraley et al., 1986). These markers for selection of plant transformation have two categories; selectable markers and scorable-assayable markers. The selectable markers include neomycin resistance, methotrexate resistance, hygromycin resistance, chloramphenicol resistance, and aminoethyl cysteine resistance. The scorable-assayable markers include opine synthesis, neomycin phosphotransferase activity, chloramphenicol acetyl transferase activity and luciferase activity.

Leaf disc transformation Because *Populus* species demonstrate high degree of developmental plasticity through tissue culture manipulation, we can regenerate whole plants through explant culture of leaf, internode, and root segments of *Populus* species. This adventitious shoot formation from cultured explants allows the utilization of the *Agrobacterium* binary vector system to transfer specific genes into *Populus* species through co-cultivation of *Agrobacterium* binary vectors with leaf discs. There are two co-cultivation methods which have become routine in some herbaceous plant species such as tobacco, tomato, petunia, cotton, and *Arabidopsis* (McCormick et al., 1986; Wei et al., 1986; An et al., 1986). One employs a nurse culture within feeder plates (Horsch et al., 1985; Lloyd et al., 1986; Fillatti et al., 1987). The use of feeder plates has some effects toward reducing the bacterial damage to the leaf pieces and stimulating

transformation events. Another co-cultivation method is direct selection after co-cultivation of *Agrobacterium* with leaf segments without placing on feeder plates (An et al., 1985; An et al., 1986; Umbeck et al., 1987; Dandekar et al., 1987).

### Conclusion

*Populus* biotechnology can offer the following potential in the near future: 1) greatly shortened tree improvement cycles for specific traits that are controlled by a mono- or oligogenes; 2) incorporation of new gene combinations by recombinant DNA methods; 3) mass micropropagation of plants with specific gene combinations through in vitro culture; 4) induction and selection of somaclonal variation for improved pest tolerance or resistance.

SECTION I. INFLUENCES OF MEDIUM CONSISTENCY  
AND SHOOT DENSITY ON *IN VITRO*  
SHOOT PROLIFERATION OF *POPULUS*  
*ALBA* X *P. GRANDIDENTATA*

Influences of medium consistency and shoot density on *in vitro* shoot  
proliferation of *Populus alba* X *P. grandidentata*\*

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## ABSTRACT

The *in vitro* shoot proliferation of *Populus alba* X *P. grandidentata* was affected by the medium consistency and shoot density, but not by three sizes of vessels. After 4 weeks of culture, the fresh weight and number of shoots per explant on liquid medium were significantly greater than those on agar-solidified medium. In particular, 3.2 shoots, 7 mm or longer per explant, were produced on liquid medium compared with 1.6 shoots per explant on agar-solidified medium. The fresh weight per explant after 4 weeks of culture on liquid medium and agar-solidified medium were 0.68 and 0.25 g, respectively. Increasing the number of shoots per vessel slowed the growth of the explants as measured by fresh weight and the number of shoots produced. There was little difference in the number of shoots produced between vessels with 1 or 2 shoots per vessel, but there were fewer shoots produced when 3 shoots were placed in each vessel.

Key words; *Populus alba* X *P. grandidentata*, shoot proliferation, micropropagation, liquid medium, shoot density.

## INTRODUCTION

For clonal propagation through *in vitro* culture of forest tree species, most of the work has concentrated on establishing optimum inorganic salt, vitamin, growth regulator and other chemical requirements. However, factors other than the chemical composition of the medium may affect the maximum shoot proliferation of tissue cultured plant material. For example, liquid medium offers advantages over agar-solidified medium, such as eliminating possible inhibitory compounds (Kohlenbach and Wernicke, 1978; Romberger and Tabor, 1971). Moreover, for commercial propagation, the cleaning of tubes is simplified, and number of explants per vessel can be increased (Harris and Stevenson, 1982; Oki, 1981; Stevenson et al., 1982). Nevertheless, *in vitro* proliferation of poplar and most other woody species has been on agar-solidified media (Ahuja, 1983; Bonga, 1982; Christie, 1978; Chun and Hall, 1984; Kim et al., 1981; Thorpe and Biondi, 1984).

The culture density within the vessel is another physical parameter that influences proliferation. Monette (1983) reported that of 3 sizes of culture vessels tested, 473 ml vessels produced significantly greater *in vitro* proliferation of grapevines than did 125 ml vessels with liquid medium.

The hybrid poplar, *Populus alba* X *P. grandidentata* has attracted much attention from several workers because of the high productivity found in naturally occurring stands and in a U.S. Forest Service trial plantation (Hall et al., 1982). However, this hybrid poplar has been limited in its potential for planting because it is difficult to root by regular greenhouse or nursery techniques. We have developed a technique for

circumventing this problem with tissue-culture techniques on agar-solidified media (Chun and Hall, 1984).

The present study was undertaken to determine the effect of liquid vs. solid medium, different vessel sizes and different initial shoot numbers per culture vessel on fresh weight and shoot number of excised shoot tips.

## MATERIALS AND METHODS

The Hansen clone of *Populus alba* X *P. grandidentata* was used in this study because of its micropropagation potential (Chun and Hall, 1984). Axillary buds were removed from the upper one third of actively growing shoots from greenhouse-grown stock plants that were 4 months old. Buds were prepared for disinfestation by trimming off leaves while retaining a small piece of petiole attached to the stem. The buds were dipped in 70% ethanol for 30 seconds before a 5-minute surface disinfestation in a solution of 0.5% sodium hypochlorite and 0.04% Tween 20 in a laminar-flow hood. This was followed with three sterile distilled-water rinses.

For the initial establishment of shoot tips in culture, excised buds, 5 to 8 mm long, were cultured on Gresshoff and Doy basal (1972) medium without hormones. This medium was adjusted to pH 5.7 after addition of 0.7% Difco Bacto-agar and then was autoclaved at 121°C for 15 minutes. Twenty milliliters of medium was aseptically dispensed into 100 x 25 mm petri dishes.

Six weeks after initiation the axillary shoots were cut into 5 to 6 segments, and transferred to proliferation medium, composed of Murashige and Skoog (1962) basal medium without kinetin and IAA, but supplemented with 0.8% Difco Bacto-agar and 1.0  $\mu$ mol (0.2 mg/l) benzyladenine (BA). After pH adjustment to 5.7 and autoclaving, 20 ml of medium was dispensed into each vessel. When the desired number of axillary shoots were produced by serial subculture, 1.5 cm long shoots were aseptically weighed and transferred to 2 media and 3 vessel sizes. The two kinds of media were proliferation medium described above with 0.8% agar (agar-solidified medium) and without (liquid medium). For each medium, the following 3

sizes of vessel were tested: 74 ml standard 100 x 10 mm plastic petri dishes (Fisher Scientific Co.), 135 ml 100 x 25 mm plastic petri dishes (Lab-Tek Products), and 233 ml Mason jars. One, 2, and 3 shoots were transferred into each vessel size and medium combination to determine the effect of shoot density. Shoots were plated cut-surface down in agar-solidified medium, and were laid flat in liquid medium. There were 4 replications for all liquid medium treatments and 6 replications for agar-solidified medium treatments. Culture vessels were covered, sealed with Parafilm and arranged in a completely randomized design on the culture bench.

Cultures were maintained at a daytime temperature of 25-28°C, with a 16 h. photoperiod and photosynthetically active radiation level of 50-60  $\mu\text{E m}^{-2}\text{s}^{-1}$  from cool-white fluorescent tubes. The nighttime temperature was controlled at 20-23°C with 8 h. darkness. Data were taken after 2 and 4 weeks of growth in the test vessels at which time each culture was blotted dry with a paper towel and weighed; shoots 3 mm or longer were counted.

## RESULTS AND DISCUSSION

Medium

The mean fresh weight of explants tested on liquid medium was significantly ( $P=0.0001$ ) more than that on agar-solidified medium after 2 and 4 weeks of culture (Fig. 1). The total mean fresh weights of explants grown on liquid medium and agar-solidified medium were 0.38 g and 0.10 g after 2 weeks and 0.68 and 0.25 g after 4 weeks of culture, respectively. The mean number of shoots produced on liquid medium was also significantly ( $P=0.0001$ ) more than that on agar-solidified medium (Fig. 2). The total mean number of shoots per explant produced on liquid medium and agar-solidified media was, respectively, 2.5 and 0.9 after 2 weeks and 5.5 and 4.0 after 4 weeks of culture. In addition the mean number of shoots 7 mm or longer in liquid medium was twice that of the number of shoots in solid medium (Table 1). This is important because in our experience, shoots 7 mm or longer become established in soil faster and start shoot elongation earlier than shorter shoots.

For *in vitro* proliferation of *Populus* species, agar-solidified media have been used exclusively (Ahuja, 1983; Christie, 1978; Chun and Hall, 1984; Kim et al., 1981). However, this experiment demonstrates that liquid medium produced more shoots with greater fresh weight than agar-solidified medium. Similar responses have been reported earlier and were shown to influence *in vitro* proliferation in apple and pear (Singha, 1982), *Picea abies* (Romberger and Tabor, 1971), and *Vitis* (Harris and Stevenson, 1982). This may be partly due to an increased nutrient availability and uptake by the shoots. Less growth and fewer shoots on agar-solidified medium have been interpreted as partly due to a lower

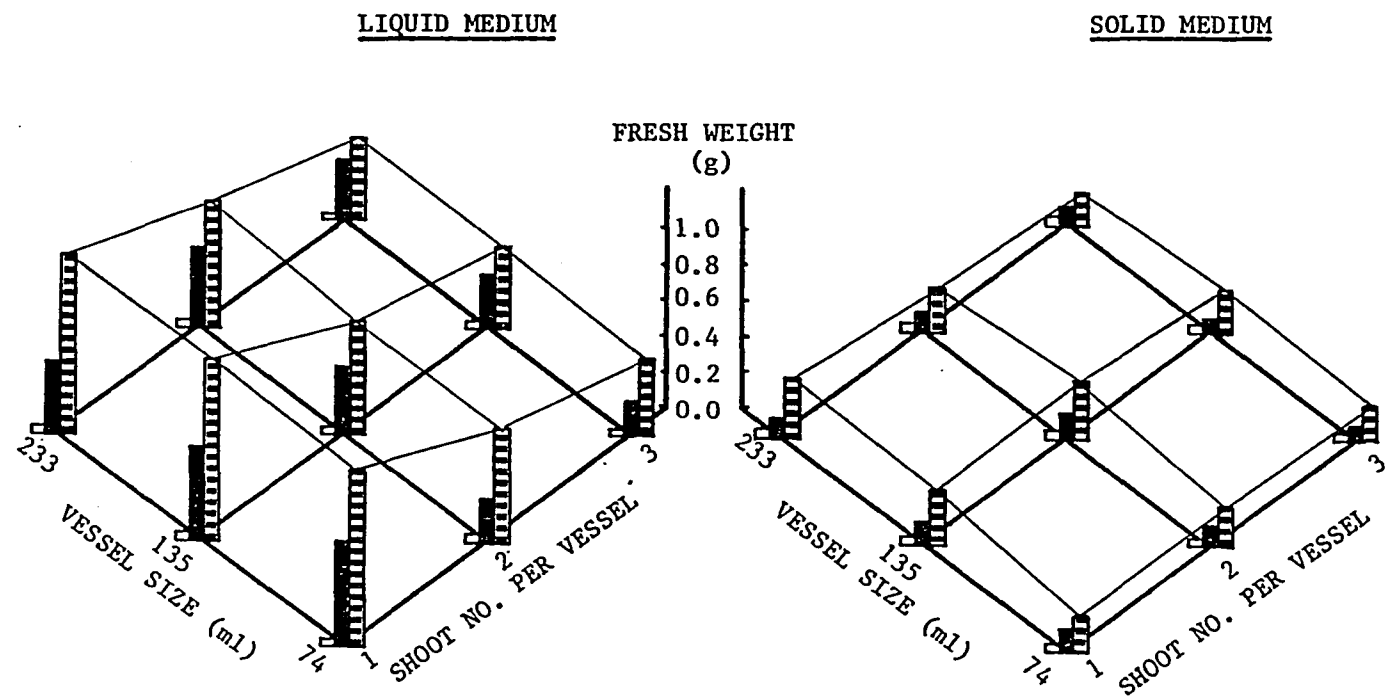


Fig. 1. Mean fresh weight at culture initiation (□), after 2 weeks (■), and after 4 weeks culture (▨)

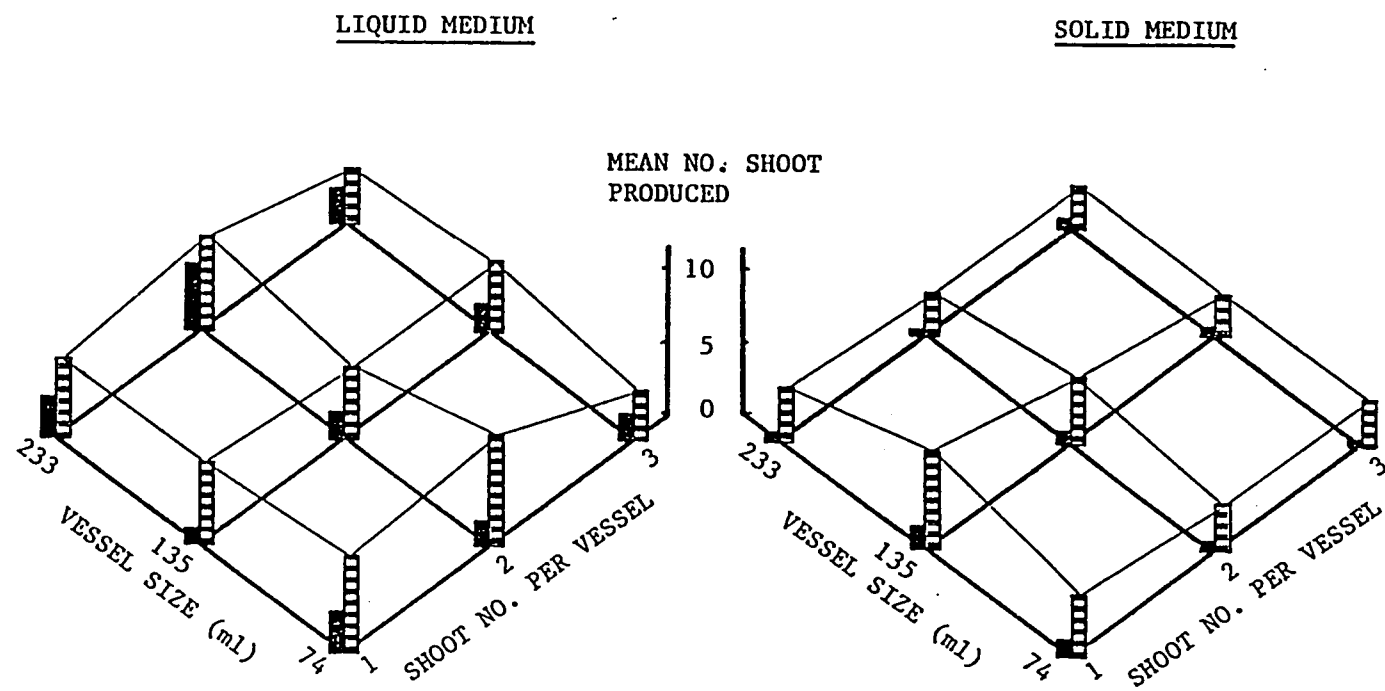


Fig. 2. Mean number of shoots per explant after 2 weeks (■) and 4 weeks culture (▨)



diffusion rate of molecules, including exuded explant metabolites through the medium (Stevenson and Harris, 1980), partly to undefined agar-borne inhibitors (Kohlenbach and Wernicke, 1978; Romberger and Tabor, 1971), and partly to a reduction of water availability to tissue growing on agar-solidified medium (Stoltz, 1971). At least in agitated liquid medium, aeration is also better than in agar-solidified medium (Kessel and Carr, 1972).

Table 1. Mean number of shoots produced after 4 weeks in 3 different sizes of vessels in liquid and solid medium

Vessel size	Liquid medium Shoot length			Solid medium Shoot length		
	Total	<7 mm	≥7 mm	Total	<7 mm	≥7 mm
74 ml	5.8	2.4	3.4	3.8	2.3	1.5
135 ml	5.2	2.2	3.0	4.8	3.1	1.7
233 ml	5.4	2.1	3.4	3.3	1.7	1.6
Mean	5.5	2.2	3.3	4.0	2.4	1.6
S.E. <sup>a</sup>	0.38	0.15	0.25	0.24	0.22	0.15

<sup>a</sup>S.E. = standard error of means.

Although the liquid medium was better than solid medium for *in vitro* proliferation, vitrification symptoms appeared in liquid medium treatments as glassiness or water logging of the tissue. Most of the vitrification started after 2 weeks of culture in liquid medium. There were no vitrification symptoms in explants grown in agar-solidified medium. In preliminary experiments, we observed that vitrification occurred more frequently on explants that were agitated on a shaker than on those in static liquid cultures. There were no significant differences among the three sizes of vessels for percent vitrification (Table 2). This is in accordance with previous reports, indicating that vitrification was not

affected by the volume of the culture containers utilized (Debergh et al., 1981; Monette, 1983).

Table 2. Percent of vitrification of explants after 2 weeks and 4 weeks of culture in liquid medium

Culture period	Size of culture vessels		
	74 ml	135 ml	233 ml
After 2 weeks	13.0	12.5	12.5
After 4 weeks	22.7	21.1	23.5

#### Vessel size and shoot density

There were no significant differences among the three sizes of vessels tested for mean fresh weight of explants after 2 and 4 weeks of culture. The total mean fresh weight of explants that were grown in 74 ml, 135 ml and 233 ml vessels were, respectively 0.20 g, 0.23 g and 0.22 g after 2 weeks of culture and 0.43 g, 0.42 g and 0.41 g after 4 weeks of culture. The total mean number of shoots produced after 2 weeks was significantly ( $P=0.05$ ) different in the 3 vessel sizes tested. However, after 4 weeks, there was no significant difference in the total mean number of shoots among the vessel sizes (Table 1). Thus, the size of culture vessel didn't influence shoot proliferation of poplar. Our results on poplar differ from those on grape, where Monette (1983) showed that there was a significant influence on fresh weight of explants and multiple shoots produced in different sizes of culture vessels.

The number of shoots cultured per vessel significantly ( $P=0.0001$ ) changed the explant growth produced over 4 weeks of culture, as measured by fresh weight and the mean number of shoots per explant. The mean number of shoots produced after 2 weeks of culture was not significantly

different. Increasing the number of shoots per vessel slowed the fresh weight accumulation (Fig. 1) and the number of shoots produced (Fig. 2). This was probably because of the competition for nutrients between shoots in the vessel. There was little difference in the number of shoots produced per explant between vessels with 1 or 2 shoots per vessel, but fresh weight was much less when 3 shoots were inoculated in each vessel. The total mean numbers of shoots produced per vessel on liquid medium were 6.2 shoots (1 shoot per vessel), 12.8 shoots (2 shoots per vessel) and 12.3 shoots (3 shoots per vessel), demonstrating that 2 shoots per vessel produced maximum shoot proliferation.

This study demonstrates that medium consistency and the number of cultured shoots per vessel have a significant influence on shoot proliferation in the Hansen clone of *Populus alba* X *P. grandidentata*. The fresh weight and the number of 7 mm shoots produced per explant after 4 weeks of culture on liquid medium were twice those of explants produced on agar-solidified medium.

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SECTION II. ISOLATION AND CULTURE OF *IN*  
*VITRO* CULTURED *POPULUS ALBA* X  
*P. GRANDIDENTATA* PROTOPLASTS

Isolation and culture of *in vitro* cultured  
*Populus alba* X *P. grandidentata* protoplasts

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## ABSTRACT

Protoplast-source material and enzyme strength had a significant influence on yield of protoplasts from hybrid poplar, *Populus alba* X *P. grandidentata*. The yield of protoplasts from *in vitro* cultured 1 month-old plantlets was more than that from greenhouse grown 4 month-old stock plant. *In vitro* cultured plantlets regularly produced more viable protoplasts with E-I enzyme solution (0.5% cellulase and 0.1% macerase) than those treated with E-II enzyme solution (1.0% cellulase and 0.2% macerase) after overnight incubation. The mean yield of protoplasts per gram fresh weight of leaves from *in vitro* cultured plantlets was  $4 \times 10^6$  with E-I enzyme solution. After 7-10 days, the cell division was first detected in these protoplast cultures. Protoplast-derived hybrid poplar cells survived over 3 weeks in culture and some continuous cell divisions were evident. Other aspects associated with protoplasts from *in vitro* cultured plantlets were also discussed.

Key word: Protoplast culture, bud culture, *Populus alba* X *P. grandidentata*



## INTRODUCTION

One of the most significant developments in plant tissue culture techniques during recent years has been the isolation, culture and fusion of protoplasts. This technique has a number of potential uses: 1) Induction of some useful somaclonal variation in protoplast cultured plants (Shepard et al., 1980; Kemble and Shepard, 1984). 2) The plasma membrane can interact with and take up macromolecules (genetic material) such as foreign organelles and organisms. So this technique can be applied to plant genetic engineering (Chun, 1985). 3) Adhesion of protoplasts can result in parasexual hybridization by protoplast fusion as with potato and tomato (Gleba and Sytnik, 1984). 4) Alleviation of ploidy level differences or sexual incompatibility problems (Redenbaugh and Westfall, 1980). 5) Single cells can be used for a mass selection system (Dodds, 1983). And finally, 6) physiological studies (Vasil, 1976).

While there are a few reports describing protoplast isolation and culture from forest tree species, it has not been possible so far to regenerate whole plants from protoplast culture of forest tree species (Ahuja, 1984). Few *Populus* species have been successfully isolated or cultured (Ahuja, 1983; Saito, 1980; Verma and Wann, 1983), and there is no report on continuous cell division and differentiation from poplar protoplasts (Ahuja, 1984).

To fully explore the potentials for protoplast technology of forest tree species, efficient reproduction methods for protoplast isolation and purification must first be established. This paper describes the isolation and culture of mesophyll protoplast from *in vitro* shoot cultured hybrid poplar plantlets, *Populus alba* X *P. grandidentata*.

## MATERIALS AND METHODS

Plant material

In vitro cultured plants of the Crandon clone of *Populus alba* X *P. grandidentata* were chosen for use in this study. The initial establishment and proliferation of *in vitro* culture of this hybrid poplar are already described in detail (Chun and Hall, 1984). Yields of protoplasts from greenhouse grown 4 month-old stock plant and *in vitro* cultured 1 month-old plantlets were compared. The disinfection method of leaves for the greenhouse grown stock plant was the same as described by Chun and Hall (1984).

Protoplast isolation and culture

Protoplasts from leaf and petiole tissue were isolated by incubating transverse sections (1 mm wide) for 15-20 h. in an enzyme solution containing E-I enzyme combination (0.5% Cellulysin cellulase and 0.1% Macerase [both from Calbiochem]) or E-II enzyme combination (1.0% Cellulysin cellulase and 0.2% Macerase), both containing 0.1% bovine albumin (Sigma) and 0.7 M mannitol in a CPW solution, at pH 5.8. The CPW salts consisted of 250 mg/l magnesium sulphate  $7H_2O$ , 100 mg/l calcium chloride  $2H_2O$ , 170 mg/l potassium dihydrogen phosphate, and 30 mg/l calcium nitrate  $4H_2O$ .

The protoplasts were purified by a combination of filtration, centrifugation and washing. The protoplasts were filtered through a 100  $\mu$  mesh and pelleted in 0.7 M mannitol + CPW solution by centrifugation at 100 x g for 10 minutes. The pellet was resuspended in a 20% sucrose + CPW solution and centrifuged at 50 x g for 10 minutes. Floating protoplasts

were collected and washed in 10% mannitol + CPW solution and plated in 2 ml of protoplast culture medium at a density of 10 to 5 X 10<sup>6</sup>/ml.

Protoplast cultures were grown on Murashige and Skoog (Murashige and Skoog, 1962) salts supplemented with thiamine HCl 0.1 mg/l, nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, glycine 2.0 mg/l, myo-inositol 100 mg/l, sucrose 30 g/l, naphthaleneacetic acid (2.0 mg/l), benzyladenine (0.5 mg/l), sucrose (30 g/l) and mannitol (90 g/l). The protoplasts were plated either in a liquid medium or embedded near the surface of a semi-solid agar medium by placing 2.0 ml of protoplast solution on 2.0 ml of melted agar medium (0.7% Difco Bacto-Agar) prior to solidification.

Cultures were maintained at a daytime temperature of 25-28°C with a 16 h. photoperiod and photosynthetically active radiation (PAR) level of 50-60  $\mu\text{E m}^{-2}\text{s}^{-1}$  from cool-white fluorescent tubes. The nighttime temperature was controlled at 20-23°C with 8 h. darkness.

## RESULTS AND DISCUSSION

*In vitro* cultured *Populus alba* X *P. grandidentata* regularly produced more viable protoplasts when treated with E-I enzyme solution than those treated with E-II enzyme solution after overnight incubation (Table 1). With over night incubation in E-I and E-II enzyme solution, mean yields of protoplasts from *in vitro* cultured plantlet were  $4 \times 10^6$  and  $0.5 \times 10^6$  per gram fresh weight of leaves, respectively. Thus the strength of enzyme solution influenced the yield of protoplasts. In some poplar species, a strong enzyme solution should be used to isolate protoplasts (Verma and Wann, 1983). In some other poplar species, only a relatively weak enzyme solution is needed to isolate protoplasts (Ahuja, 1983; Smith and McCown, 1983).

The yield of protoplasts from *in vitro* cultured plantlets was more than that from greenhouse grown plant. Generally, leaves from *in vitro* cultured juvenile plantlets and greenhouse grown stock plants provide a reasonably good protoplast-source material. However, in this experiment, insufficient protoplasts for plating were obtained from greenhouse grown stock plant. Similar results have been reported earlier and were shown to yield more protoplasts from *in vitro* cultured *Betula* and *Rhododendron* plants compared with those produced using greenhouse grown stock plants (Smith and McCown, 1983). Decreased yields and no cell division have been shown to be partly due to wrong enzyme concentrations during incubation and also partly due to the physiological growth condition of the greenhouse grown stock plants. In addition, a much lower protoplast density than optimum plating density with the greenhouse grown stock plant also caused no cell division (Table 1). For optimum growth and

differentiation, plant protoplasts should have a maximum as well as a minimum plating density (Evans and Cocking, 1977).

Table 1. Effect of different enzyme solution and material sources on protoplast yield per gram fresh weight and mitotic activity

Enzyme solution	Source of material	Yield	Mitotic activity <sup>a</sup>
E-I enzyme <sup>b</sup>	Greenhouse grown stock plant	trace	no division
	<i>In vitro</i> cultured plantlet	4 X 10 <sup>6</sup>	Second and third division
E-II enzyme <sup>c</sup>	Greenhouse grown stock plant	trace	No division
	<i>In vitro</i> cultured plantlet	0.5 X 10 <sup>6</sup>	First cell division

<sup>a</sup>Mitotic activity = observation during 3 weeks of culture.

<sup>b</sup>E-I enzyme = 0.5% cellulase and 0.1% macerage.

<sup>c</sup>E-II enzyme = 1.0% cellulase and 0.2% macerage.

Protoplasts obtained from *in vitro* cultured plantlets were spherical with well-distributed cell contents. However, there was variability in size of leaf mesophyll protoplasts from *in vitro* cultured hybrid poplar (Figure 1). This size difference was also reported by Ahuja (1983) who grouped *Populus tremula* and *P. tremuloides* protoplasts into two categories; normal and mega protoplasts. The origin of mega protoplasts is not known.

Cell wall regeneration of these protoplasts usually occurred within 48 h. Initiation of budding from protoplasts in agar-solidified medium was observed after 5-7 days in culture (Figure 2). After 7-10 days, the first cell divisions or constrictions showed in the protoplast culture (Figures 3 and 4). Cells usually enlarged and became less cytoplasmically dense during this period. Protoplast-derived hybrid poplar cells survived

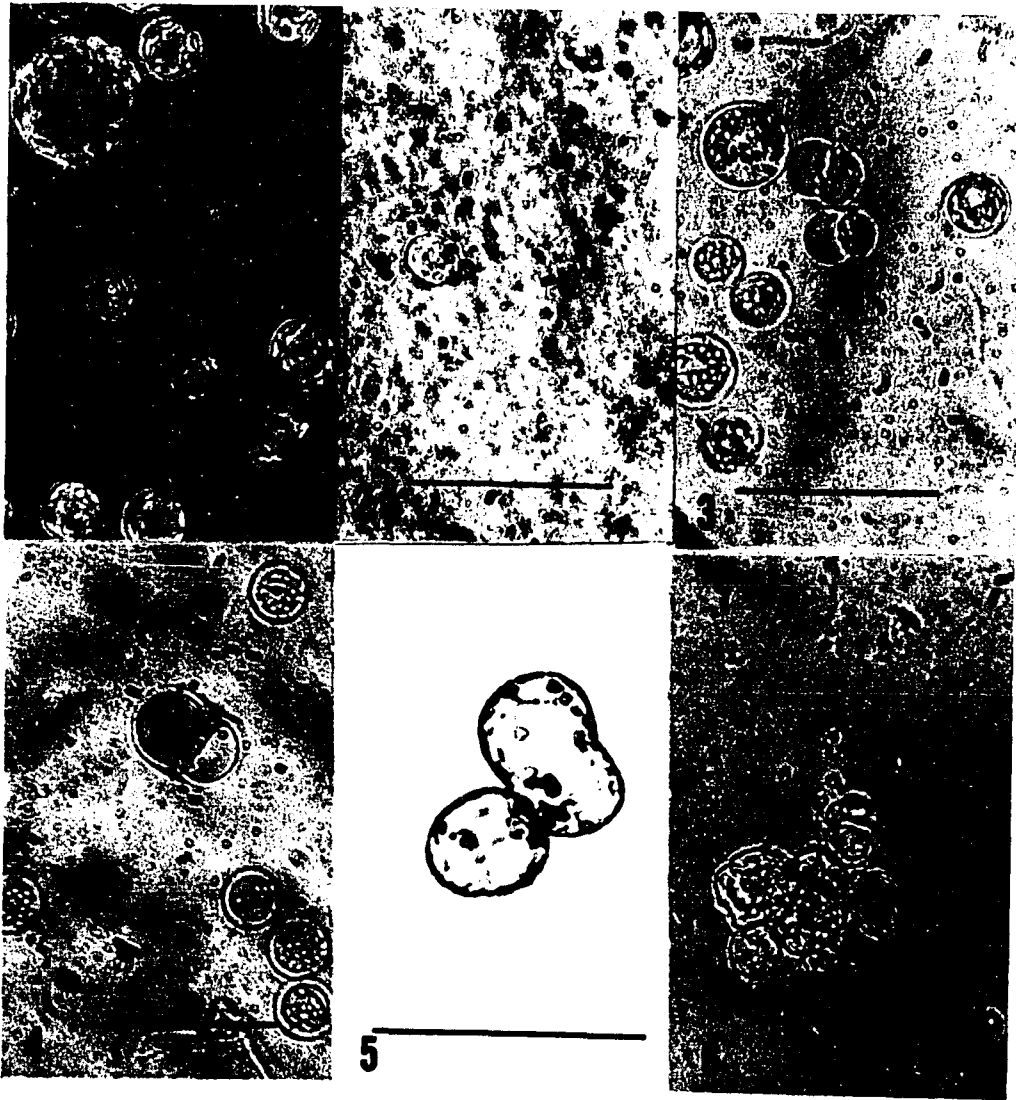
over three weeks in culture and some continuous cell divisions were evident (Figure 5). However, cell division did not continue beyond the 6-10 cell stages (Figure 6).

Even though sustained callus formation and differentiation from cultured protoplasts was not obtained in this study, it was shown that *in vitro* cultured hybrid poplar provides a very useful protoplast-source material. There are also other advantages when *in vitro* cultured plantlets are used for protoplast isolation and culture; 1) *in vitro* bud (or shoot) culture provides a genetically stable source (Lawrence, 1981), 2) juvenile growth conditions are maintained under *in vitro* conditions (Chun and Hall, 1984), 3) it provides for high morphogenic potentials for embryogenesis or organogenesis, and 4) it can also provide a year-round plant source with a cyclical growth phase in a small space.

This study clearly demonstrates that protoplast source material and enzyme strength have a significant influence on yield of protoplasts from the hybrid poplar, *Populus alba* X *P. grandidentata*. At the time of this writing, only limited attempts have been made to culture these protoplasts. More intensive studies are required on their growth and differentiation.

- Figure 1. Freshly isolated mesophyll protoplasts of *Populus alba* X *P. grandidentata*
- Figure 2. Initiation of budding in solid-agar medium after 1 week of culture
- Figure 3. First cell division in liquid medium after 1 week of culture
- Figure 4. First cell division from a mega-protoplast after 1 week of culture
- Figure 5. Second cell division
- Figure 6. Three week-old cell clusters

The scale for Figures 1-6 is 100  $\mu\text{m}$ .





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SECTION III. MORPHOGENETIC POTENTIAL OF  
LEAF, INTERNODE, AND ROOT  
EXPLANTS FROM *POPULUS ALBA* X  
*P. GRANDIDENTATA* PLANTLETS

Morphogenetic potential of leaf, internode, and root explants from  
*Populus alba* X *P. grandidentata* plantlets

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## ABSTRACT

Morphogenetic responses of explants of *Populus alba* X *P. grandidentata* plantlets depend upon the explant source and the combination of exogenously applied plant growth regulators. Leaf, internode, and root explants of two *Populus* clones, Crandon and Hansen, were different in their morphogenetic responses to various benzyladenine (BA) and naphthaleneacetic acid (NAA) combinations. Leaf explants formed the most shoots, roots, and calli, while root explants formed the least. Abaxial side culture of entire leaf explants was best suited for inducing adventitious shoots.

Key Words; *Populus* leaf culture, internode culture, root culture, morphogenetic responses, interclonal variation, BA, NAA

## INTRODUCTION

*Populus* species possess characteristics that make them well suited as a model system for studying biotechnological applications to deciduous forest-tree species (Parsons et al., 1986). Recent advances in research with *Populus* species include the introduction of DNA from genetically engineered Ti plasmids into leaf-disc explants, and the subsequent regeneration of transformed shoots (Fillatti et al., 1986). The development of regeneration techniques for *in vitro* cultured plant material is an integral part of such genetic engineering systems.

*Populus* species are among the fastest growing deciduous-tree species and are distributed throughout temperate forests in the northern hemisphere (Dickmann and Stuart, 1983). The hybrid poplar, *Populus alba* X *P. grandidentata*, has attracted considerable attention from several workers because of its high productivity in naturally occurring stands and in a U. S. Forest Service trial plantation (Hall et al., 1982). Nevertheless, its potential for planting is limited because it is difficult to root by conventional greenhouse or nursery techniques.

Two routine methods, axillary bud production (also termed axillary shoot production) and adventitious bud initiation (Faltonson et al., 1984), have been developed for experimental use with woody species. We have reported techniques for axillary bud production of *Populus alba* X *P. grandidentata* in several different media (Chun and Hall, 1984; Chun et al., 1986).

This paper addresses adventitious shoot initiation from leaf, internode, and root explants from *in vitro* cultured *Populus alba* X *P. grandidentata*. This study was conducted to determine the relation of

various combinations of BA and NAA to morphogenetic development of explants derived from *in vitro* culture.

## MATERIALS AND METHODS

Two experiments were performed. The first one (Experiment I) was conducted to examine the relation of shoot, root, and callus formation to a broad range of BA and NAA levels and to identify a relatively narrow range of BA and NAA levels that would produce maximum shoot production for the two *Populus alba* X *P. grandidentata* clones, Crandon and Hansen. Shoot production is the desired response and the most difficult one to achieve. An optimum level for shoot production is desired for explant culture. The second experiment (II) examined shoot, root, and callus formation over the narrow range identified by the first one. For both experiments, plantlets for source material were derived from axillary bud culture of two hybrid poplar clones, Crandon and Hansen. Procedures for micropropagation of these hybrid poplar clones are outlined by Chun and Hall (1984) and Chun et al. (1986). Axillary shoots were produced initially by serial subculture on MS (Murashige and Skoog, 1962) salts supplemented with thiamine HCl 0.1 mg/l, nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, glycine 2.0 mg/l, myo-inositol 100 mg/l, sucrose 30 g/l, Bacto-agar 6 g/l, and BA 0.4 mg/l at pH 5.7. Axillary shoots were transferred to rooting medium [MS + indole-3-butyric acid (IBA) 0.2 mg/l] and cultured for four weeks before serving as source material. Leaf, internode, and root explants were excised and cultured in petri dishes containing MS media with one level of BA (0, 0.1, 1.0, 2.5, or 5.0 mg/l) combined with one level of NAA (0, 0.01, 0.1, 1.0, or 5.0 mg/l). All 25 combinations of BA and NAA levels were used. In each petri dish, one internode segment (5 mm in length) and one root segment (4 cm in length) were laid horizontally on the culture medium. Six leaf segments consisting of the abaxial and



adaxial sides of 1) entire leaf with petiole, 2) terminal half segment, and 3) basal half segment were also laid flat in each petri dish (Experiment I).

For Experiment II, media containing seven levels of NAA (0, 0.01, 0.05, 0.1, 0.2, 0.5, and 1.0 mg/l) and one level of BA (1.0 mg/l), and the same kinds of explants that were used in Experiment I were tested.

There were four replications of all treatments in both experiments. Petri dishes were covered, sealed with Parafilm, and arranged in a completely randomized design within the culture chamber.

Cultures were maintained at a daytime temperature of 25-28°C, with a 16 h. photoperiod and a photosynthetically active radiation level of 50-60  $\mu\text{E m}^{-2}\text{s}^{-1}$  from cool-white fluorescent tubes. The nighttime temperature was maintained at 20-23°C during the 8 h. darkness. After five weeks of culture, regeneration was measured by recording the visible formation of adventitious shoots, roots, or calli from each leaf, internode, and root explant. The number of shoots per explant was recorded. Roots or multiple shoots originating from one locus were classified as a single regenerant.

## RESULTS AND DISCUSSION

Experiment I

The percentage of leaf, internode, and root explants producing shoots, roots, and calli are summarized in Figure 1 and Table 1 for the 25 different culture media tested. In terms of total mean frequency of all eight explants tested, adventitious shoots formed infrequently (6.9%), root formation occurred with moderate frequency (29.9%), and callus was frequently formed (72.1%) as averages for the 25 different test media. An analysis of variance (ANOVA) of the shoot formation data should not be performed because so many treatment combinations resulted in no shoot formation. Although the average percentage of callus and root formation is in a range that could be analyzed with an ANOVA, the actual treatment means vary greatly with callus formation having many 100% values and root formation having many 0% values. Therefore, these data were not analyzed with an ANOVA. Instead, the shoot, root, and callus formation data were visually examined.

For both clones, shoot formation occurred most frequently with BA concentrations of 1.0 and 2.5 mg/l with NAA from 0 to 1.0 mg/l. Abaxial side culture of leaves and internode culture were the most effective. Root formation increased with increasing NAA concentration and decreasing BA concentration. In addition, adaxial side culture of leaf explants was better than abaxial side culture for root formation, and leaf explants were better than root or internode explants. Callus formation was insensitive to BA concentration except at NAA 0 mg/l, but increased with increasing NAA concentration, particularly over the lowest three concentrations. Also, leaf and internode explants formed callus much more

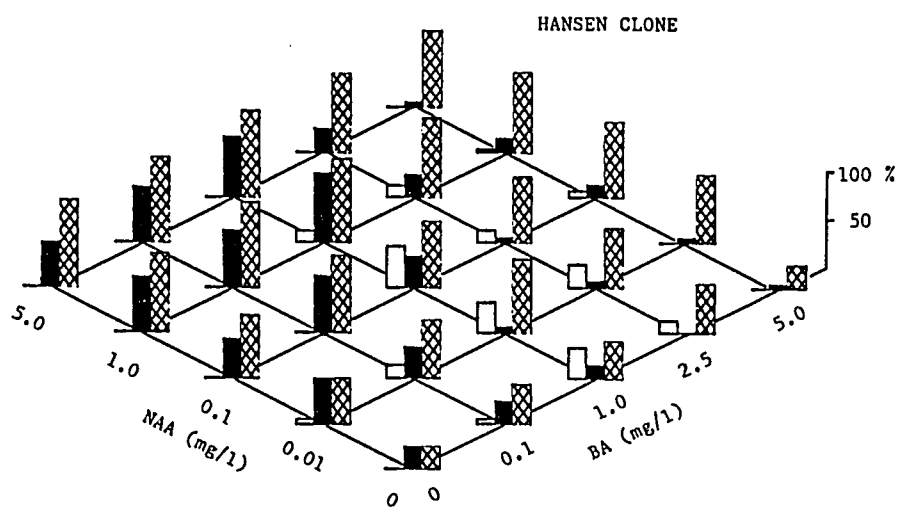
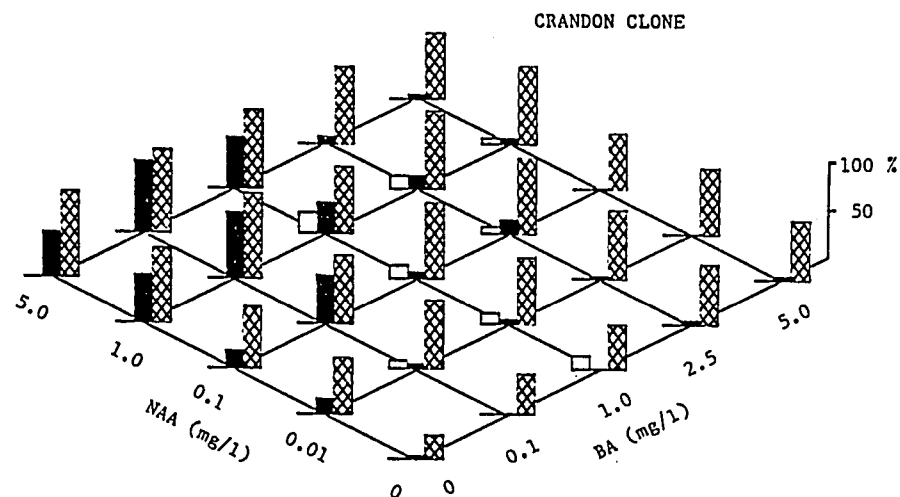


Figure 1. Frequency of shoot formation ( $\square$ ), root formation ( $\blacksquare$ ), and callus formation ( $\boxtimes$ ) on 25 different culture media (averaged over 8 explants).

often than root explants. Calli formed at the higher concentrations of BA and NAA were larger than calli formed at the lower concentrations. The Hansen clone produced more shoots, roots, and calli than the Crandon clone on most BA and NAA test combinations. Over all eight explants, the Hansen clone produced more adventitious shoots (average 1.29) than the Crandon clone (average 0.59).

Table 1. Total mean percentages of shoot, root, and callus formation for 8 different explants (averaged over both clones and all BA and NAA combinations)

Explant		Morphogenetic responses		
		Shoot form. %	Root form. %	Callus form. %
Abaxial side culture	Entire leaf with petiole	16.0	31.0	86.5
	Terminal half segment	5.5	31.0	63.0
	Basal half segment	10.0	28.0	82.5
-----				
Adaxial side culture	Entire leaf with petiole	3.5	54.5	88.0
	Terminal half segment	4.5	34.5	77.0
	Basal half segment	2.5	39.5	86.0
-----				
Internode		11.0	9.5	79.0
Root segment		2.5	11.5	72.1
-----				
Mean of all explants		6.9	29.9	72.1

## Experiment II

The data for Experiment II showed less variation among the treatments than Experiment I, and it was within the range appropriate to analyze with ANOVA. For shoot, root, and callus formation the dependent variable was whether each explant did or did not form callus, or one or more shoots, or roots. The actual values that were analyzed were 0 if there was no

formation and 1 if formation occurred. This violates some of the assumptions of ANOVA resulting in probability values that are probably too small and should be interpreted conservatively. The mean number of adventitious shoots and percentage of leaf, internode, and root explants producing shoots, roots, and calli on 7 different culture media tested are summarized in Table 2.

Table 2. Total mean number of adventitious shoots formed and percentage of morphogenetic responses on 8 different explants (averaged over 7 different media)

Explant	No. of shoot <sup>a</sup>		Shoot form. percentage		Root form. percentage		Callus form. percentage	
	Cran <sup>b</sup>	Hans <sup>c</sup>	Cran	Hans	Cran	Hans	Cran	Hans
Abaxial side culture								
Entire leaf with petiole	5.9	7.2	50.0	60.7	3.6	10.7	92.9	100
Terminal half segment	2.0	5.1	3.6	28.6	0	7.1	92.9	96.4
Basal half segment	3.0	7.0	21.4	50.0	3.6	14.3	96.4	100
Adaxial side culture								
Entire leaf with petiole	3.0	1.6	3.6	25.0	64.3	78.6	100	96.4
Terminal half segment	6.0	2.5	3.6	21.4	7.1	35.7	96.4	100
Basal half segment	2.0	3.0	3.6	32.1	39.2	57.1	100	100
Internode	2.3	4.5	10.7	46.4	0	0	89.2	100
Root segment	1.5	1.3	14.3	14.3	0	0	7.1	57.1

<sup>a</sup>No. of shoot = the average number produced by those explants that actually produced adventitious shoots.

<sup>b</sup>Cran = Crandon clone.

<sup>c</sup>Hans = Hansen clone.

Shoot formation: ANOVA showed all interactions to be significant ( $P < 0.01$ ). Examination of a plot made for each clone of percent shoot formation versus NAA concentration for the eight explants showed that for the middle concentrations of NAA, the percent shoot formation and rank for each explant varied considerably, particularly for the Hansen clone. And most of the explants formed no shoot at the lowest (0 mg/l NAA) and highest concentrations (1.0 mg/l NAA). Figure 2 shows a graph of shoot formation by concentration averaged over all 8 explants for the two clones. The Crandon clone shows almost no response to NAA concentration, while the Hansen clone shows a definite increase in shoot formation for concentrations 0.05 mg/l to 0.2 mg/l NAA. Because the responses for both clones were so different, each clone was examined individually.

The Crandon clone formed shoots on only 14% of the explants. The abaxial side of the entire leaf formed shoots 50% of the time and showed a definite response to NAA with levels 0.01 mg/l to 0.1 mg/l forming 75% or more shoots. The abaxial side culture of the basal half leaf formed shoots 21% of the time. The other 6 explants (terminal half leaf of abaxial side, three adaxial side leaves, internode, and root segment) formed shoots 15% of the time or less.

The Hansen clone formed shoots 35% of the time. The abaxial side of entire leaves was clearly the best for shoot formation (61%). The abaxial sides of basal half leaf segments (50%) and internodes (46%) were the next best explants for shoot formation. In general, abaxial side leaf cultures formed more shoots than adaxial sides. For concentrations 0.05 mg/l to 0.2 mg/l NAA, explants of three abaxial side leaves, the basal half leaf of the adaxial side, and internode averaged 60% shoot formation.

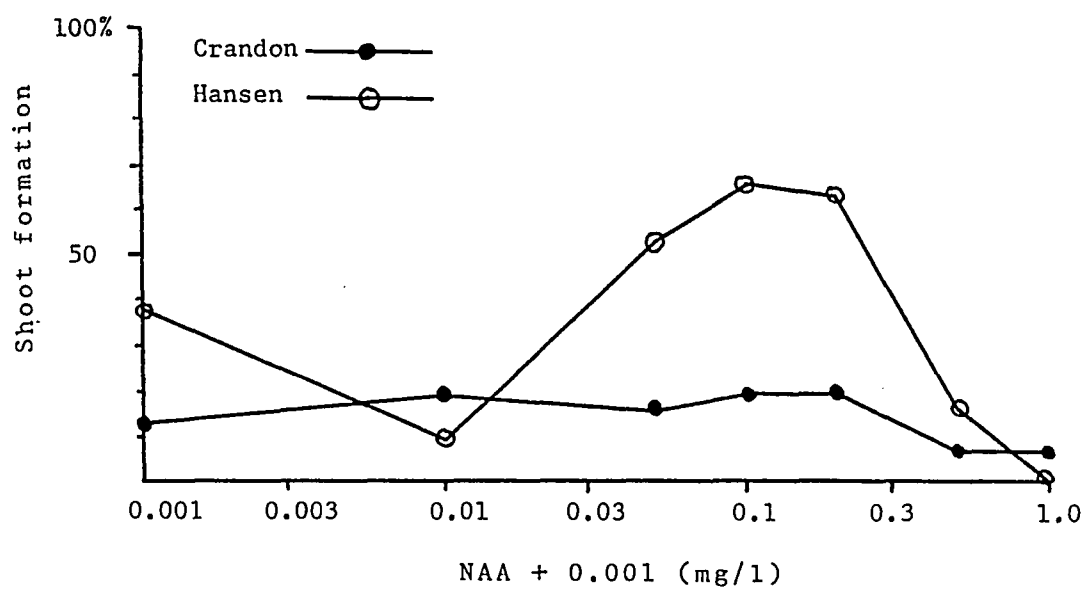


Figure 2. Mean percentage of shoot formation by concentration averaged over all 8 explants for the two clones

Root formation: Root formation had a strong NAA concentration by explant interaction ( $P < 0.001$ ). A graph of percent root formation by concentration clearly showed that the adaxial side leaf explants formed roots most frequently and that the percentage formation increased with increasing concentration of NAA (Figure 3). The abaxial side leaf explants formed no roots at concentrations 0.2 mg/l and below, and then only occasionally at the higher concentrations. The Hansen clone formed more roots than the Crandon clone ( $P < 0.01$ ), and the percentage tended to increase faster with increasing NAA concentration than that of the Crandon clone ( $P = 0.03$ ).

Differences between abaxial and adaxial side cultures could be attributed to general leaf structure. In adaxial side culture, leaf segments with midribs or petioles perhaps are not flat enough to permit sufficient contact of the cut edges with the medium. In addition, the stomata on the abaxial leaf surface may allow better absorption of exogenously applied plant growth regulators. Positional organogenesis also may involve a polarity in hormone transport and (or) endogenous hormone distribution.

Callus formation: The initial ANOVA showed a strong clone by explant interaction ( $P < 0.001$ ). A graph of the average percentage formation of callus versus explant for both clones showed the two clones were very similar in callus formation for all explants except the root segments for which the Hansen clone formed callus 57% of the time and the Crandon clone formed callus 7% of the time (Figure 4). Another ANOVA was done excluding the root segment, and there were no significant differences or interactions among any of the treatments. Both clones, all concentrations



of NAA, and all of leaf and internode explants formed callus very readily. Excluding the root segments, the Grandon clone formed callus 95% of the time, and the Hansen clone formed callus 99% of the time.

Abaxial	Entire leaf with petiole	○
side	Terminal half segment	●
culture	Basal half segment	■
-----		
Adaxial	Entire leaf with petiole	△
side	Terminal half segment	▲
culture	Basal half segment	□

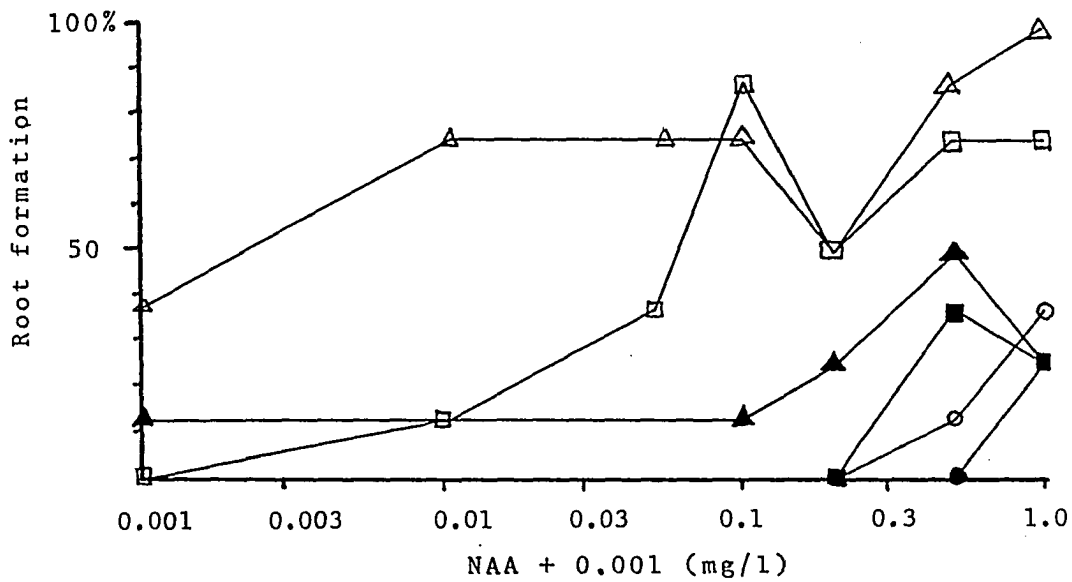


Figure 3. Mean percentage of root formation by concentration over all 8 explants of two clones

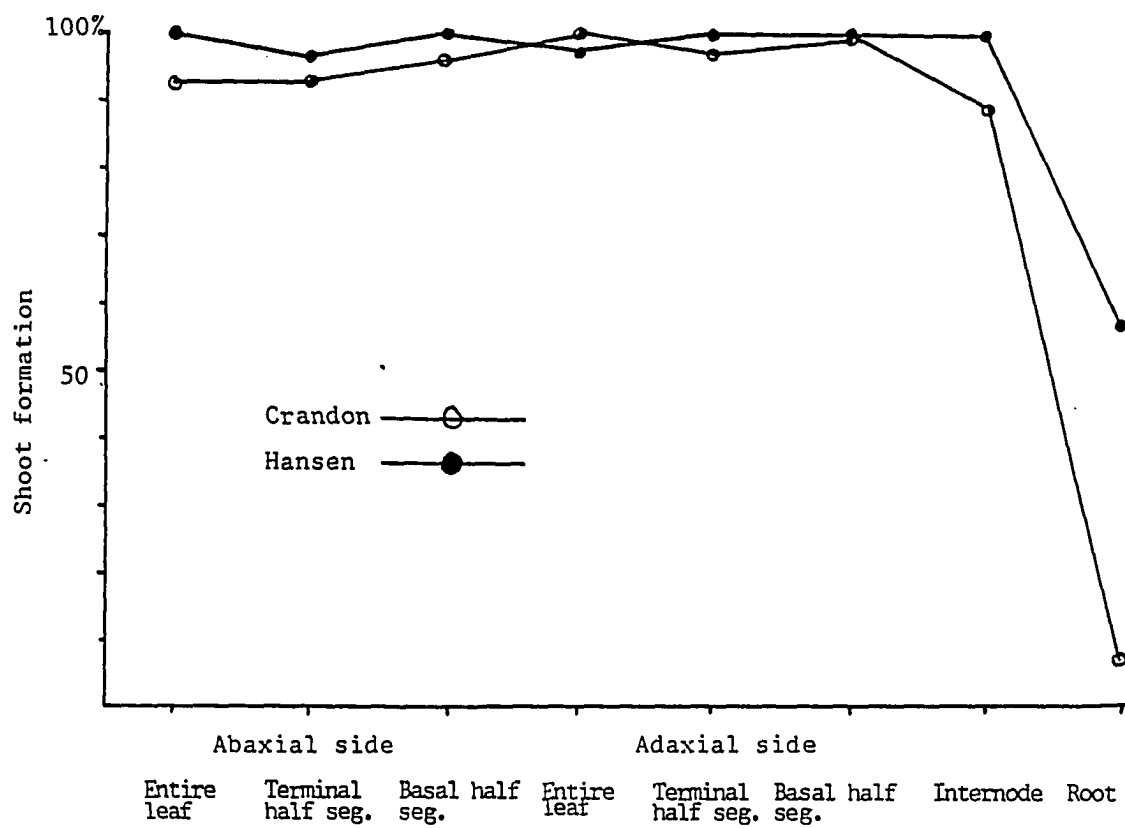


Figure 4. Mean percentage of callus formation versus all 8 explants for two clones

Number of shoots: The number of shoots produced by explants can be examined two ways, the average number produced over all explants or the average number produced by those explants that actually produced shoots. The number of shoots produced over all explants is strongly influenced by whether the explant tended to form shoots or not, but the later variable is not. Thus, the later variable allows a clearer look at what affects the number of shoots formed.

Although Crandon clone explants did not form shoots very often, when they did, they produced about as many adventitious shoots (4.1 per explant) as the explants of the Hansen clone (4.8) (Table 2). For the Crandon clone, the abaxial side for the entire leaf formed the most shoots per explant (5.9), and the rest of the explants produced about 3 shoots per explant. Also, the number of shoots produced was low for the highest and lowest NAA concentrations (19%), and higher (81%) for the other concentrations but quite variable (Table 3).

For the Hansen clone, which frequently formed shoots, the abaxial side cultures for three different leaf explants produced the most shoots per explant (average 6.4 shoots). Internodes produced 4.5 shoots per explant, adaxial side leaf explants averaged 2.4 shoots per explant, and root explants produced an average of 1.3 shoots (Table 2). Concentration of NAA had a definite effect on the number of shoots formed ( $P < 0.001$ ). The number of shoots per explant increased from NAA concentrations of 0 mg/l to 0.2 mg/l going from an average of 3.7 shoots per explant to 6.5 shoots. Then, it dropped to 1.6 at NAA concentration 0.5 mg/l and for concentration 1.0 mg/l there were no shoots formed (Table 3). Similar responses have been reported earlier for adventitious shoot formation from

cotyledon and hypocotyl cultures of *Populus tremuloides* (Wann and Einspahr, 1986).

Table 3. The mean numbers of adventitious shoots per explant by NAA concentration over all 8 explants for two clones

Clone	NAA concentration (mg/l)						
	0	0.01	0.05	0.1	0.2	0.5	1.0
Crandon	1.8	6.0	2.8	6.2	3.5	4.0	1.5
Hansen	3.7	4.3	4.0	5.5	6.5	1.6	0

Thus, the two clones of the natural hybrid poplar exhibited differences in their morphogenetic responses to explant culture. This interclonal variation in adventitious shoot regeneration from explant culture contrasts to a previous study that demonstrated little apparent interclonal variation in axillary shoot proliferation via axillary shoot culture among clones of this hybrid poplar (Chun and Hall, 1984; Chun et al., 1986). Nevertheless, differences between clones in morphogenetic responses have been reported previously for Leuce poplar (Ahuja, 1983).

The clones displayed similar developmental characteristics during adventitious shoot regeneration and followed a very specific developmental process. The most striking feature of regeneration was the strong polarity of the system. Initial adventitious shoots formed at the proximal cut surface of the petiole or leaf segment (Figure 5). After 2-3 weeks in culture, the end of the petiole or the proximal cut surface of the leaf segment became swollen, and several adventitious meristemic zones were formed (Figure 6). Adventitious shoots occasionally were generated from the marginal meristem zones on both adaxial and abaxial leaf surfaces

after four weeks of culture (Figure 7). There was no distal shoot regeneration, however, without prior initial proximal regeneration. Midribs of the leaf base or the basal petiole generated the most adventitious shoots, with shoot formation declining toward the distal regions of the leaf. Similar results have been observed with other species (Cutter, 1962; Dore, 1965).

As with leaf explant culture, polar effects also were observed in morphogenic responses of internodal explants. Callus formed first on the proximal end, before the subsequent callus formation occurred at the distal end. In contrast, adventitious shoots were formed initially at the distal end of internodal explants when internodes were cultured horizontally for 3 weeks. After 4-5 weeks in culture, adventitious shoots also were subsequently produced at the proximal end of the internodes (Figure 8). The number of adventitious shoots produced from the distal end was, however, much greater than that from the proximal end. Similar results have been reported earlier which demonstrated that 80% of the total adventitious buds were produced at the distal end of horizontally cultured internodes of *Populus trichocarpa* X *P. tacamahaca* (Douglas, 1984).

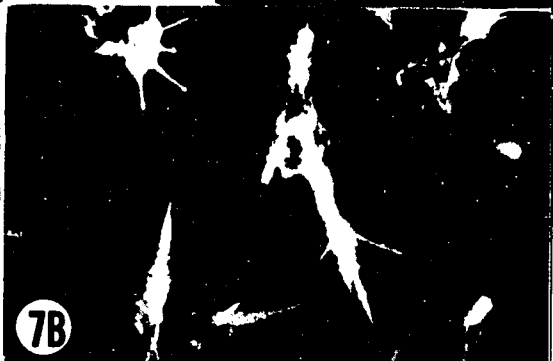
Although this hybrid poplar has good root suckering ability *in vivo*, only a few adventitious shoots were generated from *in vitro* cultured root segments (Figure 9). Our results with poplar root culture differ from those obtained previously with birch for which Srivastava et al. (1986) obtained adventitious buds that were generated from callus.

When adventitious shoots from leaf, internode, and root explant cultures were transferred onto rooting medium (MS with 0.2 mg/l IBA),

shoot elongation was evident in approximately 80% of the shoots after 1 week in culture. At the end of 4 weeks, elongation was evident in approximately 90% of the shoots. Similar responses have been demonstrated earlier with axillary bud cultures of the same clones (Chun and Hall, 1984; Chun et al., 1986).

This study demonstrates that morphogenetic responses of hybrid poplar explants depend upon the explant source and upon the combination of exogenously applied plant growth regulators. Abaxial side culture of entire-leaf explants was best suited for inducing adventitious shoots from two clones of the hybrid poplar, *Populus alba* X *P. grandidentata*. Among the three explant sources, leaf explants were the most responsive to the BA and NAA combinations tested, and root explants were the least responsive. The two clones of this hybrid poplar exhibited interclonal variations in their morphogenetic responses in leaf, internode, and root explant culture at various BA and NAA combinations. Leaf, internode, and root explant culture of *Populus alba* X *P. grandidentata* exhibit remarkable morphogenetic potential that can be readily manipulated to form shoots, roots, or calli.

- Figure 5. Six-week-old culture showing adventitious shoot regeneration on the entire leaf with petiole (left), terminal half (middle), and basal half with petiole (right) (A), and different number of adventitious shoots regenerated from three leaf explant sources (B)
- Figure 6. Two-week-old culture showing the adventitious meristem zone at the petiole (A) and proximal cut of leaf segment (B)
- Figure 7. Four-week-old culture showing the regeneration of adventitious shoots from the marginal meristem zones on both abaxial and adaxial leaf surface (A). Root formation at the end of the petiole or cut surface in adaxial side leaf culture (B)
- Figure 8. Adventitious shoot regeneration on the internode segments after six weeks of culture
- Figure 9. After four weeks of culture, adventitious shoot regeneration on a root segment





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SECTION IV. INFLUENCES OF SUBCULTURING PERIOD  
AND DIFFERENT CULTURE MEDIA ON  
COLD STORAGE OF *POPULUS ALBA* X  
*P. GRANDIDENTATA* PLANTLETS

Influences of subculturing period and different  
culture media on cold storage maintenance of  
*Populus alba* X *P. grandidentata* plantlets

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## ABSTRACT

Continuous subculture is labor intensive and requires extensive culture space. Cold storage of in vitro cultured *Populus* plantlets could serve to alleviate the maintenance requirements of an established micropropagation system, as well as provide methods to facilitate germplasm conservation. In vitro cultured hybrid poplar, *Populus alba* X *P. grandidentata* could be stored at 4°C air temperature in darkness for 24 months and still recover suitable multiplication potential. Subculturing period preceding cold storage, plantlet condition, and culturing medium all had an important influence on survival at 4°C in darkness. A one-month subculturing period preceding cold storage was better than 0-month or 2-month subculturing period preceding cold storage. Shoot proliferation medium was better than rooting medium for long term cold storage. After 24 months storage, a 70% survival percentage was obtained with plantlets possessing 4-6 axillary branching shoots that were subcultured on shoot proliferation medium for one month preceding cold storage.

Key words: *Populus*, cold storage, micropropagation, shoot culture, germplasm preservation

## INTRODUCTION

Continuous subculturing is the initial requirement for establishing a micropropagation system. Maintenance of established cultures can require substantial space, chemicals, and labor. To minimize time and expense for routine culture maintenance, preservation of plant material in a state of growth suspension has been attempted by various methods (Wilkins and Dodds, 1983). Such growth suspension methods in plant tissue culture can be divided into three categories: non-frozen cold storage, alteration of the basic culture media, and addition of growth retardants. Among these tissue culture storage methods, temperature reduction has been demonstrated to be very effective (Withers, 1985). There are many species that can be conserved by temperature reduction, especially with non-frozen, low temperature storage methods. These species include: *Malus* sp. (Lundergan and Janick, 1979), *Musa* sp. (Banerjee and Langhe, 1985), *Prunus* sp. (Marino et al., 1985), *Pinus radiata* (Aitken-Christie and Gleed, 1984), *Trifolium repens* (Bhojwani, 1981), *Allium sativum* (El-Gizawy and Ford-Lloyd, 1987) and *Populus* sp. (Chun and Hall, 1986).

The hybrid poplar, *Populus alba* X *P. grandidentata*, has attracted considerable attention from several workers because of its high productivity in naturally occurring stands and in a U.S. Forest Service trial plantation (Hall et al., 1982). Nevertheless, this hybrid poplar has been limited in its potential for planting because it is difficult to root by regular greenhouse or nursery techniques. For effective clonal propagation through *in vitro* culture of this hybrid poplar, we previously have established techniques for axillary bud culture and adventitious bud initiation from cultured explants (Chun et al., 1986; Chun, 1987), and for

survival and early growth of tissue cultured plantlets in soil (Chun and Hall, 1984). We have experienced some difficulties in the maintenance of this established micropropagation system, however, because continuous subculture requires intensive labor and considerable expense. To alleviate these problems, we attempted to determine the optimum medium and plantlet condition for non-frozen cold storage. In this paper, we report the effects of previous subculturing period and plantlet condition on maintenance of *Populus alba* X *P. grandidentata* plantlets in cold storage.

## MATERIALS AND METHODS

The Crandon clone of *Populus alba* X *P. grandidentata* was used for this experiment. Procedures for micropropagation of this hybrid poplar are outlined by Chun and Hall (1984) and Chun et al. (1986). Axillary shoots were produced initially by serial subculture on MS (Murashige and Skoog, 1962) basic salts supplemented with thiamine HCl 0.1 mg/l, nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, glycine 2.0 mg/l, myo-inositol 100 mg/l, sucrose 30 g/l, Bacto-agar 6 g/l, and benzyladenine (BA) 0.3 mg/l at pH 5.7.

Four sources of plant material were tested as follows:

1. Shootlets with 4-6 axillary branching shoots that were subcultured on MS media containing 0.3 mg/l BA for one month preceding cold storage (Figure 1).
2. Shootlets with 5-7 axillary branching shoots that were subcultured on MS media containing 0.3 mg/l BA for two months previous to cold storage (Figure 2).
3. Shoot segments, approximately 1 cm in length with one or two leaves, that were explanted on MS media containing 0.3 mg/l BA just prior to cold storage. These shoot segments were derived from plantlets grown on MS medium containing 0.2 mg/l IBA (Figure 3).
4. Plantlets that were subcultured on MS media containing 0.2 mg/l indole-3-butyric acid (IBA) for one month before cold storage (Figure 4).

For storing cultures at low temperature, each plantlet or shoot segment was transferred to a 15 x 200 mm glass culture tube which contained 15 ml medium. The culture tubes were covered with plastic caps



and sealed with Parafilm to prevent excessive drying. Eighty tubes for each of the four culture treatments were placed in a cold room, at 4°C in the dark. Every 3 months, 10 culture tubes were removed for each different culture treatment. The percentage of culture tubes with viable shoots were determined 2 weeks following removal from cold storage. To determine proliferation potential after storage, these shoots were dissected and 10 of these dissected shoots were subcultured on the same MS medium with 0.2 mg/l BA under the normal culture conditions which were controlled to maintain a daytime temperature of 25-28°C with a 16 h. photoperiod and a photosynthetically active radiation level of 50-60  $\mu\text{E m}^{-2}\text{s}^{-1}$  from cool-white fluorescent tubes. After an additional one month culture, the number of developed shoots were counted.



(A)

(B)

(C)

Figure 1. Shootlets with 4-6 axillary shoots on shoot proliferation medium for one month subculturing period preceding cold storage (A), and after 12 months (B), and 24 months (C) cold storage



(A)

(B)

(C)

Figure 2. Shootlets with 5-7 axillary shoots on shoot proliferation medium for one month subculturing period before cold storage (A), and after nine months (B), and 12 months (C) cold storage

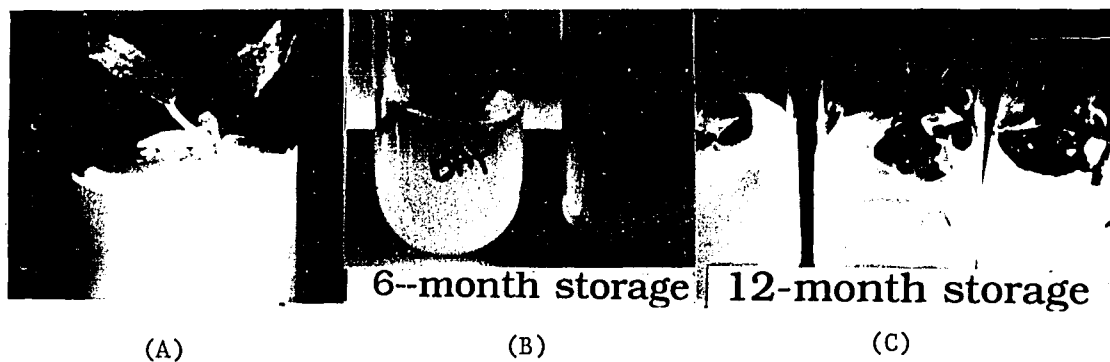


Figure 3. Shoot segment on shoot proliferation medium just prior to cold storage (A), and after six months (B), and 12 months (C) cold storage

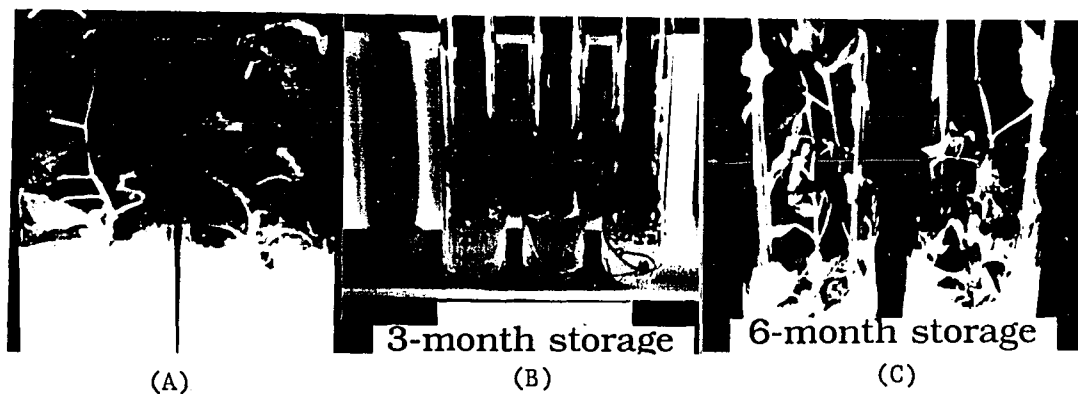


Figure 4. Plantlets with 1-2 shoots on rooting medium for one month subculturing period before cold storage (A), and after three months (B), and six months (C) cold storage

## RESULTS AND DISCUSSION

The effects of previous subculturing period and medium on survival and shoot proliferation of the *Populus alba* X *P. grandidentata* Crandon clone after cold storage are summarized in Table 1. During the first 12 months of cold storage, plantlets that were subcultured on rooting medium (MS+IBA 0.2 mg/l) for 1 month before cold storage and shoot segments with one or two leaves that were placed on shoot proliferation medium (MS+BA 0.3 mg/l) immediately prior to cold storage exhibited similar survival tendencies. The survival of both cultures decreased with the increased duration of cold storage. They showed only 30% survival after 12 months cold storage. With shootlets subcultured on shoot proliferation medium for 2 months prior to cold storage, 50% of the cultures survived after 12 months cold storage; however, these cultures showed a rapid decrease in their viability and vigor as cold storage periods increased beyond 12 months. The shootlets that were subcultured on shoot proliferation medium for 1 month before cold storage obtained the highest survival percentage and also did not change in their viability and vigor until after 12 months of cold storage. The survival percentage and multiplication potential of these cultures began to decrease after 15 months cold storage with the survival percentage of these shootlets dropping to 70%. Among the cultures that were subcultured on shoot proliferation medium for varying periods before cold storage, a 1 month subculture period prior to cold storage generated the highest survival percentage and multiplication potential. The previous subculturing period is, therefore, a determining factor in culture survival during cold storage, and in subsequent culture proliferation potential. Our results with *Populus* are in accordance with

those with *Prunus* where Marino et al. (1985) reported that of three different previous subculturing period, 14 days of previous subculturing period resulted in significantly better survival and regenerative capacity than did 0 or 7 days of previous subculturing period. Lundergan and Janick (1979) also demonstrated that with a previous subculturing period, shoot tips of apples (*Malus domestica*) could be stored for one year.

Table 1. Effects of previous subculturing period and culture medium on survival and shoot proliferation of *Populus alba* X *P. grandidentata* plantlets

Storage period (month)	Shoot proliferation med. <sup>a</sup>						Rooting med. <sup>b</sup>	
	0 month		Previous subculturing period				1 month	
	Surv. <sup>c</sup> %	Shoot no.	Surv. %	Shoot no.	Surv. %	Shoot no.	Surv. %	Shoot no.
3	80	1.4	100	7.3	90	5.3	80	3.6
6	80	1.6	100	6.5	70	4.2	50	3.2
9	60	1.2	100	6.8	60	3.8	50	3.2
12	30	1.3	100	6.4	50	3.8	10	3.1
15	20	1.5	80	6.6	20	4.2	10	3.1
18	0		90	4.6	0		0	
21	0		80	4.4	0		0	
24	0		70	4.3	0		0	
S.E. <sup>d</sup>		0.13		0.26		0.20		0.15
Mean		1.40		5.86		4.26		3.34

<sup>a</sup>Shoot proliferation med. = MS + BA 0.3 mg/l.

<sup>b</sup>Rooting med. = MS + IBA 0.2 mg/l.

<sup>c</sup>Surv. % = Survival percentage.

<sup>d</sup>S.E. = Standard error of means.

The shootlets with axillary branching shoots that were subcultured on shoot proliferation medium for one month preceding cold storage were not affected in their potential for rapid shoot multiplication with storage periods up to 15 months. After 15 months of cold storage, however, the

average shoot proliferation rate decreased from 6.7 to 4.4 shoots. In contrast, the rate of shoot proliferation shootlets subcultured for 2 months on shoot proliferation medium and plantlets subcultured for 1 month on rooting medium appeared to remain consistent throughout all storage periods. Cultures with one or two months previous subculture on shoot proliferation medium produced more multiple shoots than did those with previous subculture on rooting medium. Similar responses have been reported in an earlier study in which the number of axillary shoots induced from axillary branching shoots that were subcultured on shoot proliferation medium was more than that from axillary buds subcultured on rooting medium (Chun and Hall, 1984). This result is perhaps attributable to preconditioning of the plantlets that were previously subcultured on a cytokinin medium before cold storage. The shoot segments that were explanted on shoot proliferation medium just before cold storage produced only a few axillary branching shoots after cold storage because these shoot segments were also derived from plantlets grown on rooting medium.

In relation to other aspects of cold-stored plantlets, the shootlets which were subcultured for 1 month on shoot proliferation medium before cold storage had small, green leaves and healthy stems until 9 months of cold storage. After 12 months of cold storage, the shootlets had developed elongated, thin stems and small, yellowish leaves (Figure 1). The lower leaves on stems in these cultures became necrotic after 12 months of cold storage. From this time, etiolated stems with only a few yellowish leaves were observed. After 24 months of cold storage, these etiolated and elongated stems turned green when returned to normal culture conditions. Stem and leaf necrosis started to occur after 3 to 6 months

of cold storage on plantlets in three other treatments (Figures 2-4). This damage may be due in part to an accumulation of oxidative substances in the medium due to defoliation and topping of stored plantlets and also to the limited availability of nutrients for the plantlets which were subcultured for two months before cold storage (Druart, 1985).

Our cold storage test was conducted in complete darkness because there is evidence that complete darkness is more suitable for successful cold storage of *in vitro* cultures of *Prunus* rootstocks than 16 h. photoperiod with high light intensity (Marino et al., 1985). However, Preil and Hoffmann (1985) have demonstrated that low level illumination (10-50 lux) is much more effective than high level illumination (500 lux) for successful longterm storage of *Chrysanthemum* at 2-3°C. In contrast, Mix (1985) reported that the potato varieties could be stored with high illumination during a 16 h. photoperiod for 3 years.

This study demonstrates that *in vitro* cultured hybrid poplar plantlets can be preserved at low temperatures. Because clones of this important hybrid poplar are routinely grown in greenhouses as a source of germplasm, this relatively simple and inexpensive cold storage method could reduce the costly greenhouse maintenance of these poplar stocks. The technique could also facilitate germplasm shipments, as well as alleviate the maintenance problems of established micropropagation systems.

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SECTION V. *IN VIVO* AND *IN VITRO* POLYPLOIDY  
INDUCTION IN THE CRANDON CLONE OF  
*POPULUS ALBA* X *P. GRANDIDENTATA*

*In vivo* and *in vitro* polyploidy induction  
in the Crandon clone of *Populus alba* X *P. grandidentata*

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## ABSTRACT

Grandon clone of *Populus alba* X *P. grandidentata* was treated with various colchicine concentrations and treatment durations to determine the best method for inducing polyploidy through *in vivo* and *in vitro* treatment of axillary buds. Colchiploid shoots were only produced from *in vivo*, but not from *in vitro* colchicine treatments. Most colchiploid shoots originated from buds that were treated 24 h. duration with 1% colchicine solution and 48 h. duration with 2% colchicine solution. Twelve colchiploid shoots produced from *in vivo* colchicine treatment continued to exhibit the chimera leaf shape after cuttings and have mixed chromosome numbers, 2n (38) and 4n (76). The adventitious shoots produced from chimeric leaf discs through *in vitro* culture appeared either normal or colchiploid leaf shape with thicker stem diameter. The colchiploid adventitious shoots had the mixed 2n-4n chromosome numbers.

Key words: *Populus*, colchiploid, tetraploid, colchicine, chimera

## INTRODUCTION

Since the first practical method of doubling chromosomes in plants by colchicine treatment was reported in 1937 (Blakeslee and Avery, 1937), colchicine treatment has been widely used for inducing polyploidy in forest tree species. These include various pines (*Pinus* sp.) (Mirov and Stockwell, 1939; Hyun, 1954; Mergen, 1959), giant sequoia (*Sequoiadendron giganteum* Lindl.) (Jensen and Levan, 1941), Japanese cypress (*Chamaecyparis obtusa* Endl.) (Kanezawa, 1951), European larch (*Larix decidua* Mill.) and Norway spruce (*Picea abies* (L.) Karst) (Illies, 1952), balsam fir (*Abies balsamea* (L.) Mill.) (Mergen and Lester, 1961), birches (*Betula* sp.) (Johnsson, 1956), Siberian elm (*Ulmus pumila* L.) (Dermen and May, 1966), and aspens (*Populus* sp.) (Mattila, 1961; Einspahr, 1965). Even though colchicine treatment provides a convenient and practical way of doubling chromosome number in most herbaceous plant species, woody species have proven to be more recalcitrant to colchicine treatment. There are several methods of colchicine application. These methods involve the treatment of the seeds, axillary buds, shoots, or seedlings with one of the four types of colchicine media: 1) an aqueous solution, 2) a glycerine solution, 3) agar, or 4) a lanoline paste (Dermen, 1940). Problems with colchicine treatment of woody plants include: 1) polyploid tissues throughout a plant are not uniformly obtained by colchicine treatment, consequently a chimera may result, and 2) competition may occur between polyploid and nondoubled tissue and the doubled tissues are displaced by the faster dividing diploid cells. To overcome these problems and to utilize the advantages of *in vitro* systems, colchicine treatment and selection has been done *in vitro* for blueberries (*Vaccinium*

sp.) (Lyrene and Perry, 1982; Goldy and Lyrene, 1984), and several herbaceous species such as potato (*Solanum bulbocastanum* Dun.) (Weatherhead and Henshaw, 1979), barley (*Hordeum vulgare* L.) (Subrahmanyam and Kasha, 1975) and tobacco (*Nicotiana tabacum* L.) (Murashige and Nakano, 1966; Burk et al., 1972). *In vitro* colchicine treatment and selection have the following advantages: 1) studies can be conducted year-round in a small space, 2) there is a higher morphogenetic potential for organogenesis, 3) a large number treatments and selections can be made, and 4) subculturing can be used to isolate tissue and produce plants with the desired ploidy level.

The importance of polyploid induction in treespecies for forest tree improvement has been stressed by Wright (1976), Einspahr (1984), and Hall (1985) among others. However, perfect polyploid induction of woody species by colchicine treatment has had limited success (Mattila, 1961; Einspahr, 1965). *Populus* species are promising candidates as breeding targets of polyploidy because earlier works demonstrated rapid growth, large size, and superior wood and pulp quality of triploid aspens (*Populus tremuloides* Michx., Müntzing, 1936) (*Populus tremula* L., Einspahr, 1965; 1984).

In this paper, we report the results of *in vivo* and *in vitro* polyploidy induction in the Crandon clone of *Populus alba* X *P. grandidentata*.

## MATERIALS AND METHODS

Experiment 1: *In vivo* colchicine treatment of axillary buds

Shoot cultures of *Populus alba* X *P. grandidentata* Crandon clone were grown *in vitro* on the modified MS medium (Murashige and Skoog, 1962) as previously described Chun and Hall (1984), and Chun et al. (1986). One hundred shoots about 2 cm in length were rooted in potting medium, and then acclimatized under intermittent mist in a shaded greenhouse for 4 weeks. Plastic cups 10 cm in diameter x 15 cm in length, were use as pots. Beginning in the fifth week after transplanting, the plantlets were placed on a culture bench in the greenhouse. A combination of incandescent and fluorescent lighting provided an 18 h. photoperiod for the plantlets during the experiment. Beginning in the fourth week after transplanting, Peters water soluble fertilizer (20-20-20) was supplied twice a week. Seven weeks after transplanting, the plantlets were transplanted into potting medium in standard 15 cm round pots. Ten weeks after the initial planting, 80 plantlets were top pruned to approximately 30 cm to 60 cm in height.

Colchicine solution was applied to the four uppermost axillary buds on the plantlets. The remaining axillary buds were removed. Colchicine solutions of 1% or 2% in 10% aqueous glycerine were used. One to three applications of colchicine were repeated every 24 h. or 48 h. to determine the effect of application interval. In addition, two different axillary bud manipulations were tested. For the first method, the top half of the axillary bud was cut off horizontally and removed. For the second method, after the top half of the bud was cut off horizontally, the lower half of the bud was split vertically to the meristem. A small drop of the

colchicine solution was placed on the cut surfaces of the axillary buds using a syringe. The control plants were prepared in the same manner as those treated, but no colchicine solution was applied. The pots were arranged as 2 replicates of a completely randomized block design with a pot serving as the experimental unit. Two weeks after the first colchicine treatment, the number of shoots that exhibited retarded growth and distorted leaf shape was recorded as an indication of colchicine treatment effect (Dermen and Diller, 1962; Dermen and May, 1966). After 2 more weeks, leaves that had initiated after the colchicine treatment were fully expanded. The shoots with leaves that exhibited putative polyploid characteristics in whole or in part, such as unusually large leaves, exceptionally dark green leaves, or thickened leaves with thicker than normal veins, were selected. Two cuttings of each of the selected shoots were rooted into potting medium and placed under intermittent mist in a shaded greenhouse. Three weeks after cutting, 4 root tips from each cutting were harvested for chromosome counting.

#### Experiment 2: *In vitro* colchicine treatment of axillary buds

Axillary shoots culture of *Populus alba* X *P. grandidentata* Crandon clone were established. Procedures for micropropagation of this hybrid poplar clone are outlined by Chun and Hall (1984) and Chun et al. (1986). Axillary shoots were initially produced by serial subculture on shoot proliferation medium containing MS basic salts, thiamin HCl 0.1 mg/l, nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, glycine 2.0 mg/l, myo-inositol 100 mg/l, sucrose 30 g/l, agar 6 g/l and benzyladenine (BA) 0.3 g/l at pH 5.7. Axillary branching shoots about 1 cm in length were



harvested from vigorously growing shootlets grown on shoot proliferation medium. These axillary shoots were placed in petri dishes of shoot proliferation liquid medium (MS+BA 0.3 mg/l, but without agar) containing various colchicine concentrations (0.01%, 0.025%, 0.05%, and 0.1%). After 24, 48, 72, and 96 h. of colchicine exposure, the explants were rinsed three times in distilled water and planted on shoot proliferation agar medium for regeneration of axillary branching shoots from treated shoots. Ten treated shoots were planted into test tubes for each treatment duration-colchicine concentration combination.

Cultures were maintained at a daytime temperature of 25-28°C with a 16 h. photoperiod and a photosynthetically active radiation level of 50-60  $\mu\text{E m}^{-2}\text{s}^{-1}$  from cool-white fluorescent tubes. The nighttime temperature was maintained at 20-23°C during the 8 h. darkness. After 6 weeks of culture, each test tube was checked for the presence of shoots with increased diameter or abnormal leaf shape.

### Experiment 3: *In vitro* adventitious shoot induction from colchiploid leaf discs

Two selected colchiploid chimeric plants derived from experiment 1 were used for micropropagation via axillary bud culture. These two chimeric plants were found to be 2n-4n chimeras by root tip chromosome counting and continued to exhibit chimeric leaf characteristics after rooting. To produce adventitious shoots from leaf explants of the two chimeric plants (designated here No. 12 and No. 23), the axillary buds were removed from the upper one third of actively growing shoots of each chimeric plant. The micropropagation procedure was the same as in

Experiment 2. After two serial subcultures on shoot proliferation medium, the axillary shoots of the chimeric plants were transferred to rooting medium (MS+indole-3-butyric acid (IBA) 0.2 mg/l) and cultured for four weeks before serving as source material. For adventitious shoot regeneration from leaf discs of chimeric plantlets, modified MS medium containing BA 1.0 mg/l and naphthalene acetic acid (NAA) 0.1 mg/l was used. The procedure for leaf disc culture is outlined by Chun (1987). After five weeks of culture, regeneration was measured by recording the visible formation of adventitious shoots. These adventitious shoots were rooted in potting medium to count root tip chromosome number.

#### Preparation of root tip squash chromosome counts

To count root tip chromosomes, a modified Palmer and Heer's (1973) root tip squash technique for counting soybean chromosomes was used. One cm of the root tip was harvested in the morning for pretreatment. The root tips were pretreated in covered vials with a saturated paradichlorobenzene (PDB) solution at 15°C for 2 h. The pretreatment was followed by fixation (for 24-48 h.) in Farmer's fluid (3:1, absolute ethyl alcohol - glacial acetic acid). The material was then hydrolyzed at 60°C in 1N HCl for 10 min. Hydrolysis was followed by staining with Feulgen solution for one and a half h. and then by soaking in cold tap water for 20 min. After staining and cold treatment, the material was placed in Pectinase (Sigma; 9.1 units/mg) solution for 1 h. at 30°C. The root tips were then refrigerated in 70% ethanol in corked vials.

Less than 1 mm of root tip was ground on a slide with a glass rod in a drop of propio-carmin stain. After squashing with a cover slip, the slide was pressed firmly under filter paper.

## RESULTS AND DISCUSSION

Experiment 1: *In vivo* polyploidy induction

The mean percentages of shoot development from axillary buds that exhibited retarded growth and distorted leaf shape by various colchicine treatment methods is summarized in Table 1. The 24 h. treatment duration produced more shoots from colchicine treated-buds (88%) than the 48 h. duration (69%) ( $P=0.003$ ) two weeks after treatment. Shoot development was more with the 1% colchicine solution (84%) than with 2% colchicine solution (72%) ( $P=0.03$ ). One application of colchicine produced more shoots (95%) than two (80%) or three applications (59%) ( $P=0.001$ ). Three applications at both colchicine concentrations was toxic and resulted in the loss of axillary buds. Of the two axillary bud manipulation methods, method 1 (top half of the axillary bud removed by horizontal incision) was less harmful to shoot development (88%) from axillary buds than method 2 (top half of bud removed, lower half of bud split vertically to meristem) (69%) ( $P=0.003$ ).

Table 1. Percentage of shoot development from axillary buds after various colchicine treatment

Colchicine conc.	1%						2%					
	Method 1 <sup>a</sup>			Method 2 <sup>b</sup>			Method 1			Method 2		
Appl. freq. <sup>c</sup>	1	2	3	1	2	3	1	2	3	1	2	3
Duration												
24 h <sup>d</sup>	100	100	75	100	88	88	100	100	75	100	100	25
48 h <sup>e</sup>	100	100	88	75	50	50	100	75	38	88	25	38

<sup>a</sup>Method 1 - axillary bud manipulation method 1.

<sup>b</sup>Method 2 - axillary bud manipulation method 2.

<sup>c</sup>Appl. - application frequency.

<sup>d</sup>24 h - every 24 h. treatment.

<sup>e</sup>48 h - every 48 h. treatment.

Shoots of treated axillary buds developed more slowly by several days to weeks than the untreated buds. There was a visual difference between treated and non-treated shoots in the early leaf flushing stage. Young leaves of treated shoots exhibited darker green color, greater leaf thickness, thicker veins, and more pubescence than untreated leaves (Figure 1). The treated shoots initially produced about 3-5 leaves which exhibited retarded growth and distorted leaf shape, the indicators of colchicine effect (Figure 2). This colchicine effect has been reported earlier on various colchicine-treated woody species such as *Ulmus pumila* (Dermen and May, 1966), camellias (*Camellia* sp.) (Ackerman and Dermen, 1972), chestnuts (*Castanea* sp.) (Dermen and Diller, 1962), grape (*Vitis vinifera* L.) (Dermen, 1954), and tetraploid hybrid aspen (*Populus tremula* X *P. tremuloides*) (Mattila, 1961). Five weeks after treatment, most leaves which initiated after treatment appeared more structurally normal. However, some leaves possessed putative polyploid characteristics. The putative polyploid leaf shape characteristics can be divided into three categories: 1) irregular marginal teeth, thicker veins, darker green color, and had varying pubescent densities on the adaxial side (Figure 3); 2) one half of the leaf or part of the leaf exhibited the characteristics described in 1) (Figure 4); and 3) the entire leaf appeared slightly shorter and broader with a darker green color and thicker veins than untreated leaves (Figure 5). The number of leaves with these polyploid characteristics on a shoot varied widely (one to all). The appearance of a broad sectorial or periclinal form in later-developing leaves implies that some cells of the shoot apex have been polyploidized (Dermen and Diller, 1962; Mattila, 1961).



Figure 1. Young leaves of colchicine-treated shoots exhibited darker green color, greater leaf thickness, thicker veins, and more pubescence (on the left) than untreated leaves (on the right) two weeks after colchicine treatment



Figure 2. The basal leaves of a shoot grown from a colchicine-treated bud exhibiting retarded growth and distorted leaf shape



Figure 3. Putative polyploid leaf which appeared to have irregular marginal teeth, thick veins, darker green color (middle), and had varying pubescence densities on the adaxial side (right), and normal leaf (left)



Figure 4. One half or part of the leaf which had irregular teeth, thick veins, and a darker green color

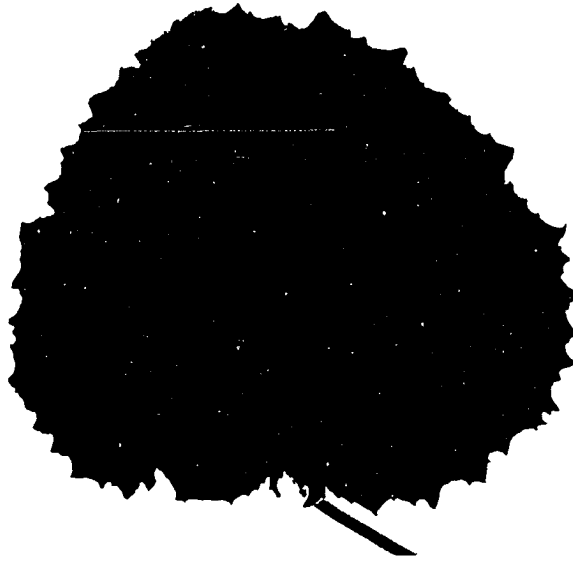


Figure 5. Putative polyploid leaf which appeared slightly shorter and broader with a darker green color and thick veins

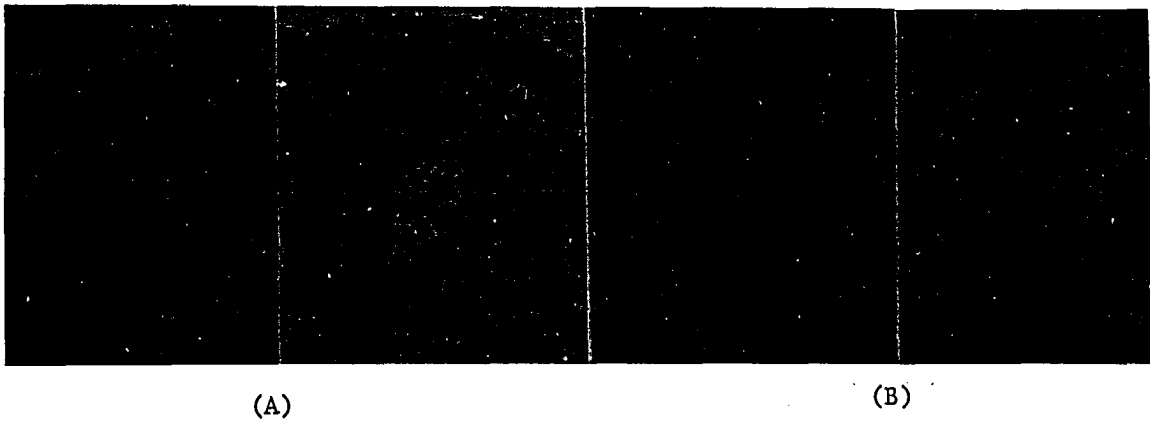


Figure 6. Approximately 76 chromosomes (A) and 38 chromosomes (B) from *in vivo* colchicine treatment of axillary buds



A total of 17 putative colchiploid shoots from 12 different treatments were observed among the 150 shoots which developed after colchicine treatment. The percentage of shoots which bore putative polyploid leaves from colchicine treatment is summarized in Table 2. The percentages of putative colchiploidy shoots which appeared after treatment every 24 h. with 1% colchicine solution for 1 to 3 applications ranged from 13% to 29%. There were no colchiploid shoots from the 48 h. treatment with 1% colchicine concentration. In contrast, more putative colchiploid shoots formed after the 48 h. treatment cycle using one to three applications of 2% colchicine (ranging from 12.5% to 66.7%). There was no correlation between the frequency of shoot development from treated buds and the appearance is real the cause is putative colchiploid appearance of those shoots. Even though the loss of colchicine treated buds increased with increasing application frequency, there was a much higher chance of obtaining putative colchiploid shoots from the surviving

Table 2. Percentage of colchiploid shoots among the shoots which exhibited colchicine effects

Colchicine conc.		1%						2%					
Appl. freq. <sup>c</sup>	Duration	Method 1 <sup>a</sup>			Method 2 <sup>b</sup>			Method 1			Method 2		
		1	2	3	1	2	3	1	2	3	1	2	3
24 h <sup>d</sup>		0	12.5	16.7	12.5	14.3	28.6	0	0	33.3	0	0	50.0
48 h <sup>e</sup>		0	0	0	0	0	0	12.5	16.7	66.7	42.9	50.0	0

<sup>a</sup>Method 1 = axillary bud manipulation method 1.

<sup>b</sup>Method 2 = axillary bud manipulation method 2.

<sup>c</sup>Appl = application frequency.

<sup>d</sup>24 h = every 24 h. treatment.

<sup>e</sup>48 h = every 48 h. treatment.

buds under the higher application frequencies.

Two cuttings from each of 12 putative colchiploid shoots from each treatment were rooted to obtain root tip chromosome counts. Most cuttings continued to bear chimeric leaves, ranging from a few to all of the leaves. However, there were also eight cuttings (four colchiploid shoot sources) which exhibited normal leaf shape. Reversion of polyploid characteristics to normal leaf shape has previously been reported (Dermen and May, 1966; Dillieu, 1967a). This may be due to displacement of the polyploid section by the fast-dividing diploid cells.

Because *Populus* species have a small amount of nuclear DNA [about 1.7 picogram in 2n cell (Dhillon, 1987)] and high chromosome number ( $2n=38$ ), their chromosomes are very difficult to separate in cell squashes. For this reason, accurate counting are difficult. Even though the method we used does not always give an exact count, we can identify the cells as probable diploid or tetraploid. Because of this difficulty, root tip chromosome counts were repeated. All cuttings which continued to produce the putative chimeric leaves were mixed 2n-4n colchiploids (Figure 6). In some cases, all root tips of a cutting had 4n chromosomes. When the chromosome countings were later repeated from the same cuttings, however, some root tips were mixed 2n-4n, or only 2n. This is in accordance with a previous report, indicating that 2n or 4n chromosome numbers were observed from root tips of *Populus tremula* X *P. tremuloides* which were originated from seeds treated with 0.1% colchicine solution (Mattila, 1961).

Experiment 2: *In vitro* colchicine treatment of axillary buds

Survival rate of *in vitro* treated shoots decreased with increasing colchicine treatment duration and concentration (Table 3). There is evidence that shoot cultures treated *in vitro* with colchicine generate three different types of axillary branching shoots. These include: 1) normal shoots, 2) mixtures of normal shoots and thick shoots as chimeras, and 3) all thick shoots or pure polyploid (Lyrene and Perry, 1982). Therefore, we used these criteria for selecting putative polyploid shoots. Concentrations of colchicine for our experiment were chosen according to the published results of Pery and Lyrene (1984), and Goldy and Lyrene (1984). Even though several thick shoots appeared with the higher concentrations and longer durations of colchicine, these thick shoots reverted to the normal diameters after one or two subcultures. No other morphological anomalies, such as an increase in pubescence on the adaxial surface, a darker green color, thicker leaves, or altered leaf shape, were exhibited in these thick shoots. Some thick shoots were rooted for chromosome counting. All shoots counted had normal  $2n$  chromosome numbers. Our results on *Populus* differ from those on *Vaccinium* sp., in which

Table 3. Effects of colchicine concentration and treatment duration on survival rate (%) of treated explants

Colchicine conc. (%)	Duration (h.)				Mean
	24	48	72	96	
0.01	100	100	100	100	100
0.025	100	100	100	100	100
0.05	90	90	90	80	90
0.1	100	90	80	90	90
Mean	97.5	97.5	92.5	92.5	

Perry and Lyrene (1984) and Goldy and Lyrene (1984) showed that colchicine concentrations between 0.01% and 0.05% were adequate for *in vitro* polyploid induction. *Vaccinium* sp. is the only genus of woody plants where polyploid induction through *in vitro* colchicine treatment has been successful (Lyrene and Perry, 1982; Goldy and Lyrene, 1984; Perry and Lyrene, 1984). This result seems to reflect different responses between *Populus* and *Vaccinium* species to *in vitro* colchicine treatment.

Experiment 3: *In vitro* adventitious shoot induction from colchiploid leaf discs

Initial axillary bud cultures of No. 12 and No. 23 chimera shoots produced in Experiment 1 generated axillary shoots which exhibited colchicine-derived morphological anomalies. After two subcultures of these shoot tips on shoot proliferation medium (MS+BA 0.3 mg/l), two different types of shootlets were generated. In the first, shootlets appeared normal; in the second, shootlets had thick shoots with chimeric leaves. These thick shoots continued to bear the colchiploid leaves when cultured on rooting medium (MS+IBA 0.2 mg/l) (Figure 7).

Results from chimera leaf disc cultures of No. 12 and No. 23 explants are summarized in Table 4. Chimeric leaf culture of No. 12 explant exhibited a higher frequency of adventitious shoot formation (14.3%) than the No. 23 leaf explants (5.8%). However, the untreated leaf culture exhibited a higher frequency of adventitious shoot formation (33.3% vs. 10.5%), and more shoots were produced per leaf explant (4.4 vs. 1.9) than with No. 12 or No. 23 leaf cultures. This may be due in part to the different number of serial subcultures between the untreated and treated



Figure 7. Plantlet which exhibited thick stem diameter and possesses chimeric leaves (middle and left), and normal plantlet (right) 4 weeks after culture on rooting medium

Table 4. Number of adventitious shoots formed from 2 colchiploidy and untreated leaf disc culture on regeneration medium (MS+BA 1.0 mg/l+NAA 0.1 mg/l) after 4 weeks of culture. The data are combined from 2 separate experiments

Leaf explant source	No. 12	No. 23	Control
No. of total leaf discs cultured	56	86	24
Leaf no. which formed shoots	8	5	8
Shoot formation frequency (%)	14.3	5.8	33.3
Total no. of adv. shoots produced	13	11	44
Normal shoots no.	11	10	44
Colchiploid shoots no.	2	1	

explants and to the structural changes of colchiploid chimeric leaves. Two different adventitious shoot morphologies, normal and colchiploid shoots, were formed from leaf disc culture of these two explants. Two colchiploid adventitious shoots were selected from the 13 shoots produced from the No. 12 explant, and one colchiploid adventitious shoot was selected from the 11 shoots produced from the No. 23 explant. All colchiploid shoots and five of each normal appearing shoots were rooted for chromosome counting. All colchiploid shoots had mixed  $2n-4n$  chromosome numbers and all shoots that appeared normal had  $2n$  chromosome numbers. An adventitious shoot, however, may initiate as a homogeneous diploid or tetraploid, or as a diploid/tetraploid cytochimera from a chimeric leaf. Three different ploidy conditions ( $2n$ ,  $2n-4n$ , and  $4n$ ) of adventitious shoots from chimeric leaves have been reported in earlier studies using herbaceous species such as *Nicotiana tabacum* (Dulieu, 1967b; Opatrny and Landa, 1974), tomato (*Lycopersicum peruvianum* Mill.) (Sree Ramulu et al., 1976), *Peperomia obtusifolia* (Bergann and Bergann, 1982) and geranium cultivars (*Pelargonium* sp.) (Cassells and Minas, 1983). In contrast, Burk (1975) demonstrated that leaves of colchicine induced haploid/diploid cytochimeras of *Nicotiana tabacum* produced only either haploid or diploid adventitious shoots. At present, success in regenerating tetraploid adventitious shoots from *in vitro* cultured chimeric leaf explants remains limited. In the future, identification and histological classification of ploidy levels in chimeric leaves and more intensive adventitious shoot induction from *in vitro* chimeric leaf explants will be conducted.

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SECTION VI. TRANSFORMATION OF *POPULUS*  
SPECIES BY AN *AGROBACTERIUM*  
BINARY VECTOR SYSTEM

Transformation of *Populus* species by an *Agrobacterium*  
binary vector system

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## ABSTRACT

Three clones of *Populus alba* X *P. grandidentata* have been tested as hosts for *Agrobacterium tumefaciens* strains A281 and A348. We determined the optimum concentration of kanamycin sulfate for effective selection of leaf disc-derived, transgenic tissues transformed using *Agrobacterium* binary vector pGA472 containing a neomycin phosphotransferase gene which confers kanamycin resistance. Of the two *Agrobacterium* strains, A281 strain containing pTiBo542 appears to be well suited to serve as a helper plasmid for binary vector systems. A relatively low kanamycin sulfate concentration (10 mg/l) inhibited adventitious shoot initiation from leaf discs on regeneration medium. Transformed kanamycin resistant calli were obtained by culturing *Agrobacterium* inoculated leaf discs on selective regeneration medium. The transformed kanamycin resistant calli continued to grow on the regeneration media supplemented with kanamycin sulfate to levels of 50 and 200 mg/l. However, the non-co-cultivated control calli were severely inhibited on the regeneration medium containing 50 mg/l kanamycin sulfate.

Key Words: transformation, *Populus*, binary vector, NPT-II gene, genetic engineering

## INTRODUCTION

Since the first successful plant transformation was reported in 1983 (Murai et al., 1983; Herrera-Estrella et al., 1983), *Agrobacterium*-mediated gene transfer methods have been developed for several important forest tree species such as *Populus* (Fillatti et al., 1987), *Pinus taeda* (Sederoff et al., 1986), and *Pseudotsuga menziesii* (Dandekar et al., 1987). *Agrobacterium*-mediated gene transfer methods utilize the natural ability of *Agrobacterium tumefaciens* to infect many dicotyledonous and gymnospermous plants (De Cleene and Delay, 1976). The transfer DNA (T-DNA) of the Ti plasmid is transferred to and stably incorporated into the nuclear DNA of cells transformed during the infection process (Chilton et al., 1977; 1980; Willmitzer et al., 1980). For detailed discussions on the development of *Agrobacterium*-mediated gene transfer methods, the reader is referred to recent reviews by Fraley et al. (1986) and Perani et al. (1986).

The construction of *Agrobacterium* binary vector systems was promoted by the recent discovery that the T-DNA and the virulence (*vir*) region of *Agrobacterium tumefaciens* Ti plasmid could be separated onto two different plasmids without losing the transfer capability of the T-DNA. Binary vector plant transformation systems consist of two plasmids in *Agrobacterium*: the binary vector plasmid and a helper plasmid (Hoekema et al., 1983; An et al., 1985). A typical binary vector contains several useful characteristics such as: 1) T-DNA border sequences which are required for successful transfer of DNA from the *Agrobacterium* cell into the plant genome. 2) A wide host range replicon which can replicate in both *Escherichia coli* and *Agrobacterium* cell systems. The desired foreign

gene is cloned into the engineered binary T-DNA plasmid between the border sequences. 3) A selectable marker gene that usually confers antibiotic resistance to allow selection of transformed plant material. The typical helper Ti plasmid is an intact wild type or a disarmed (T-DNA deleted) Ti plasmid that usually contain supervirulent (broad host range) *vir* genes. The *vir* genes on a helper Ti plasmid act in *cis* to promote the transfer of the T-DNA on its own plasmid or in *trans* to transfer the T-DNA on the binary plasmid (Hille et al., 1984). Nevertheless, the ability to regenerate plants from leaf segments remains as the critical limitation toward obtaining transgenic plants for most *Agrobacterium* host species.

*Populus* species and hybrids are promising candidates as recipients for Ti plasmid-mediated gene transfer (Parsons et al., 1986; Fillatti et al., 1987; Klopfenstein et al., 1987). Earlier work reports the susceptibility of various *Populus* species to infection by naturally-occurring *Agrobacterium tumefaciens* (De Cleene and Deley, 1976). Phytohormone independent callus growth has been obtained from stem and shoot segments of *Populus trichocarpa* X *P. deltoides* following transformation by wild type *Agrobacterium tumefaciens* strains A281 and A348 (Parsons et al., 1986). Modified T-DNA containing a glyphosate-resistance gene has been incorporated into hybrid *Populus* and expressed (Fillatti et al., 1987).

In this preliminary study, we report on 1) the host range of *Agrobacterium tumefaciens* strains A281 and A348 on three clones of *Populus alba* X *P. grandidentata*; 2) the optimum concentration of kanamycin for effective selection of leaf disc-derived, transgenic tissue of this hybrid poplar that expresses a transferred neomycin phosphotransferase gene; and

3) transformation of hybrid poplar cells with a neomycin phosphotransferase gene.

## MATERIALS AND METHODS

Agrobacterium host range study

The host range of *Agrobacterium* strain A281 and A348 were evaluated with three clones (Crandon, Hansen, and Sherrill) of *Populus alba* X *P. grandidentata*. To inoculate the three clones of this hybrid poplar with strains A281 and A348, four shoots about 2 cm in length from axillary shoots growing *in vitro* on MS medium (Murashige and Skoog, 1962) containing basic salts, thiamin HCl 0.1 mg/l, nicotinic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, glycine 2.0 mg/l, *myo*-inositol 100 mg/l, sucrose 30 g/l, agar 6 g/l and benzyladenine (BA) 0.3 mg/l (pH 5.7) of each clone were rooted under intermittent mist in a shaded greenhouse. After four weeks of acclimatizing process, these rooted cuttings were placed in a growth chamber and maintained at a daytime temperature of 25-28°C, with 16 h. photoperiod and a photosynthetically active radiation level of 50-60  $\mu\text{E m}^{-2}\text{s}^{-1}$  from cool-white fluorescent tubes.

To prepare cultures for inoculation of plant tissues, single colonies of each *Agrobacterium* strain were isolated and cultured in liquid MGL medium (2.5 g/l yeast extract, 5 g/l tryptone, 5 g/l NaCl, 5 g/l mannitol, 1.16 g/l monosodium glutamate, 0.25 g/l  $\text{KH}_2\text{PO}_4$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 mg/l biotin at pH 7.0) (An, 1986). The bacterial cultures were grown overnight (to O.D. 600 = c.a. 1.2) in a shaking incubator at 28°C. Plants were inoculated by syringe puncture. Approximately 5-10 inoculations were made per plant stem at 1 cm intervals from the shoot tip. Tumor formation was scored four weeks after inoculation.



Bacterial strains

Wild type *Agrobacterium* strains (A281 and A348) and two *Agrobacterium* strains (6044 and 6048) containing a binary plasmid vector (pGA472) were obtained from Dr. M. P. Gordon at the University of Washington and Dr. G. An at Washington State University, respectively. Although the bacterial strains 6044 and 6048 contain the same binary T-DNA plasmid (pGA472), they each contain a different chromosomal background and different helper plasmids (Table 1). Strain 6044 contains a wild type, supervirulent (broad host range) Ti plasmid (pTiBo542) as the helper plasmid, while strain 6048 contains a disarmed (T-DNA deleted) Ti plasmid (pAL4404). Binary vector pGA472 contains T-DNA borders, a chimeric gene containing a nopaline synthase promoter and the coding sequences for neomycin phosphotransferase (NPT), the ColE1 replicon, bacteriophage lambda *cos* site and wide host range replicon (An et al., 1985). The two wild type strains were grown on MGL medium at 28°C, and two bacterial strains that contained a binary vector pGA472 were grown on MGL agar medium containing 20 mg/l kanamycin sulfate and 10 mg/l tetracycline HCl. Liquid cultures were grown overnight with shaking in the same MGL with antibiotics for

Table 1. *Agrobacterium* strains and plasmids used for hybrid poplar transformation

<i>Agrobacterium tumefaciens</i> strain	Chromosomal background	Ti plasmid	Binary vector	Selectable markers
A281	C58	pTiBo542	-	-
A348	C58	pTiA6	-	-
6044	C58	pTiBo542	pGA472	NOS-NPTII
6048	LBA4404	pAL4404	pGA472	NOS-NPTII

binary vector *Agrobacterium* and without antibiotics for wild type strains at 28°C.

#### Kanamycin concentration test

To determine the optimum concentration of kanamycin for effective selection of transgenic tissue, regeneration media containing nine different kanamycin concentrations (0, 10, 20, 30, 40, 50, 100, 150, and 200 mg/l) were tested. The regeneration media was MS medium with BA 1.0 mg/l, and naphthaleneacetic acid (NAA) 0.1 mg/l at pH 5.7 as previously described (Chun, 1987). Three clones of *Populus alba* X *P. grandidentata* were tested. Six leaf explants per petri dish were cultured to determine the morphogenetic responses on the five different kanamycin media. These petri dishes were arranged as three replicates of a completely randomized block design with a petri dish serving as the experimental unit. Culture room conditions were similar to those during the inoculation stage of cuttings. Four weeks after culturing, regeneration was measured by recording the visible formation of adventitious shoots, roots, or calli from each leaf disc.

#### Plant tissue culture and regeneration

Leaf explants were obtained from plantlets derived from axillary bud culture of three *Populus alba* X *P. grandidentata* clones. The initial establishments and proliferation of *in vitro* bud culture of this hybrid poplar have been previously described (Chun and Hall, 1984; Chun et al., 1986). Axillary shoots were produced initially by serial subculture on MS medium with BA 0.4 mg/l (pH 5.7). For adventitious shoot regeneration from leaf discs, MS medium containing BA 1.0 mg/l and NAA 0.02 mg/l was used for the Crandon clone, and BA 2.5 mg/l and NAA 0.02 mg/l was used for

the Hansen and Sherrill clones. Leaf explants (entire leaf, terminal half, and basal half segments) were placed in abaxial and adaxial side culture and monitored for shoot, root, and callus formation.

#### Plant transformation studies

Three clones of *in vitro* cultured *Populus alba* X *P. grandidentata* were used in this study. A binary vector system was utilized for transforming the hybrid *Populus* clones by co-cultivation of leaf segments with *Agrobacterium* strains 6044 and 6048. The basic transformation and selection system used with *Populus* is similar to that used with tobacco (An et al., 1986). Following a 3-day co-cultivation, leaf segments were washed in liquid MS salts, then cultured for seven days on shoot regeneration medium containing cefotaxime sodium salt (250 mg/l) and carbenicillin disodium salt (500 mg/l) to prevent growth of *Agrobacterium*. In subsequent transfers, kanamycin sulfate (60 mg/l) was incorporated into the culture medium to select for regeneration of transformed shoots.

## RESULTS AND DISCUSSION

Host range study

The results of the *Agrobacterium* host range study are summarized in Table 2. A different response was observed between the two strains of *Agrobacterium tumefaciens*. Although both A281 and A348 strains have the same chromosomal background of naturally occurring strain C58, strain A281 exhibited an overall higher virulence on the *Populus* clones than did strain A348. This is probably due to the supervirulent (broad host range) Ti plasmid (pTiBo542) that strain A281 possesses (Hood et al., 1984; An et al., 1985). Strain A348 contains the Ti plasmid pTiA6. These results are in accordance with a previous report (Parsons et al., 1986), in which A281 produced tumors on greenwood stems of *P. trichocarpa* X *P. deltoides*, but A348 did not. Klopfenstein et al. (1987) also demonstrated that *Agrobacterium* strain A281 exhibited an overall higher virulence on the various *Populus* species and hybrids than did strain A348. Tumor formation was observed 1-2 weeks after inoculation (Figure 1). Four weeks after inoculation, most tumors ranged in size from 1 to 3 mm in diameter. Opine analysis and culturing of tumors from this experiment was not attempted. The supervirulent Ti plasmid (pTiBo542) of *A. tumefaciens* strain A281 appears to be well-suited to serve as a helper plasmid for binary vector systems designed to transform the three clones of *P. alba* X *P. grandidentata*.

Kanamycin concentration test

The effect of kanamycin concentration on the morphogenetic responses by leaf discs of *Populus* clones is summarized in Table 3. A relatively low level (10 mg/l) of kanamycin sulfate inhibited shoot initiation from

Table 2. Host range of *Agrobacterium tumefaciens* strains A281 and A348 on three clones of *Populus alba* X *P. grandidentata*

Species	Clone	% tumor formation <sup>a</sup>	
		A281	A348
<i>P. alba</i> X <i>P. grandidentata</i>	Crandon	25	0
	Hansen	10	0
	Sherrill	50	0

<sup>a</sup>% tumor formation = percent tumor formation was calculated as the number of tumors on two plants divided by the number of inoculation sites (5-10/plant) times 100.



Figure 1. Gall formation on a two month-old *P. alba* X *P. grandidentata* plantlet inoculated four weeks earlier with *A. tumefaciens* strain A281

Table 3. Morphogenetic responses of leaf explants on regeneration media (BA 1.0 mg/l + NAA 0.1 mg/l) containing various concentrations of kanamycin sulfate

Kanamycin conc.	Morphogenetic responses <sup>a</sup>	Grandon	Hansen	Sherrill
0 mg/l	Callus form.	78.6 (%)	92.9 (%)	90.5 (%)
	Shoot init.	23.8	54.8	9.5
	Root init.	9.5	28.6	0
10 mg/l	Callus form.	33.3	70.8	45.8
	Shoot init.	0	0	0
	Root init.	16.7	37.5	8.3
20 mg/l	Callus form.	16.7	25.0	16.7
	Shoot init.	0	0	4.2
	Root init.	12.5	12.5	0
30 mg/l	Callus form.	25.0	37.5	45.8
	Shoot init.	0	0	0
	Root init.	16.7	16.7	16.7
40 mg/l	Callus form.	25.0	29.2	25.0
	Shoot init.	0	0	0
	Root init.	16.7	16.7	16.7
50 mg/l	Callus form.	14.3	11.9	14.3
	Shoot init.	0	0	0
	Root init.	7.1	7.1	4.8
100 mg/l	Callus form.	0	0	16.7
	Shoot init.	0	0	0
	Root init.	0	0	0
150 mg/l	Callus form.	0	5.6	0
	Shoot init.	0	0	0
	Root init.	0	0	0
200 mg/l	Callus form.	5.6	0	0
	Shoot init.	0	0	0
	Root init.	0	0	0

<sup>a</sup>Morphogenetic responses = morphogenetic responses of leaf explants were measured as the percentage of leaf explants producing shoots, roots, and calli.

leaf discs of the three clones of this hybrid poplar. Higher kanamycin sulfate concentrations (50-100 mg/l) were required to prevent callus formation. Callus often developed on the parts of leaves that were not in direct contact with the medium, but died when subcultured on the shoot regeneration media containing 60 mg/l kanamycin sulfate. Even though root formation at the proximal end of leaf explants was observed frequently at lower kanamycin concentrations (10-50 mg/l), these roots were initiated only on adaxial side cultures and did not contact the medium. Because the morphogenetic responses of these hybrid *Populus* clones are very sensitive to kanamycin, this antibiotic should serve as an effective agent to select for transgenic tissue that is expressing a transferred neomycin phosphotransferase (NPT II) gene.

Because the NPT II gene frequently is used as a dominant marker for selecting transformed plant cells, kanamycin is one of the most frequently used antibiotics for transformation studies (Fraley et al., 1986). Various *Agrobacterium* T-DNA binary vectors contain the neomycin phosphotransferase gene which encodes resistance to the kanamycin through detoxification by phosphorylation.

With regard to *Populus* species, Fillatti et al. (1987) have demonstrated that 60 mg/l of kanamycin was an effective concentration of the selective antibiotic for selection of transformed shoots.

#### Transformation of leaf discs

Attempts to transfer a neomycin phosphotransferase gene to *P. alba* X *P. grandidentata* clones resulted in the formation of callus in regeneration media containing 60 mg/l kanamycin sulfate. Leaf discs

transformed with binary vector 6044, which contains a wild type supervirulent (broad host range) Ti plasmid as the helper plasmid, produced large quantities of kanamycin-resistant callus on the edges of each leaf disc. Transformed kanamycin-resistant calli were first visible 1-2 weeks after placing the leaf discs on selective medium. These kanamycin resistant calli grew rapidly when transferred to fresh regeneration medium containing kanamycin. The percentages of callus formation by the three clones of the hybrid poplar are listed in Table 4. Sherrill and Crandon clones produced kanamycin-resistant callus at a much higher frequency than did the Hansen clone.

Table 4. Comparison of binary vector 6044 (wild type Ti helper plasmid) and 6048 (disarmed Ti helper plasmid) transformation on regeneration media containing 60 mg/l kanamycin. Callus formation was scored after 4 weeks of co-cultivation

		Crandon	Hansen	Sherrill
Strain 6044	No. shoot produced	0	6	0
	No. leaf pieces with callus	8/26 (30.8%)	6/36 (16.7%)	10/32 (31.3%)
Strain 6048	No. shoot produced	0	3	0
	No. leaf pieces with callus	1/39 (2.6%)	2/35 ( 5.7%)	0/43 ( 0%)

Leaf discs transformed with binary vector 6048, which contains a disarmed (T-DNA deleted) Ti plasmid (LBA4404), produced only a few calli on one leaf of the Crandon clone and on two leaves of the Hansen clone in areas that were not in direct contact with the selective medium. When these calli were transferred to the regeneration medium containing



kanamycin, all calli died. Similar responses have been reported in an earlier study in which kanamycin-resistant shoots were not recovered from explants of *Populus* co-cultivated with a binary vector containing LBA4404 as a helper plasmid (Fillatti et al., 1987). Although leaf discs of the Hansen clone co-cultured with the binary vectors 6044 and 6048 produced six and three shoots, respectively, on the shoot regeneration media containing 60 mg/l kanamycin, these shoots did not grow on the shoot proliferation media (MS+BA 0.3 mg/l) containing 60 mg/l kanamycin sulfate.

The putatively transformed calli were cut into small pieces and aseptically weighed. These small callus pieces were then transferred to media (MS+BA 1.0 mg/l and NAA 0.1 mg/l) containing 0, 50, 100, 150, and 200 mg/l kanamycin sulfate. The calli of the Sherrill clone were used only for comparisons of callus growth between callus putatively transformed with binary vector 6044 and control callus derived from non-co-cultivated explants. The callus putatively transformed with the binary vector continued to grow at all test levels of kanamycin (Figure 2). A statistical contrast was done between growth of calli grown on 0 mg/l and those grown on all other levels of kanamycin tested for the putatively transgenic calli and non-co-cultivated control calli. The contrast for the putatively transgenic calli showed no difference ( $P=0.9174$ ), but for the non-co-cultivated control calli, the contrast showed a highly significant difference ( $P=0.0016$ ). The non-co-cultivated control calli were severely inhibited by 50 mg/l kanamycin sulfate (Table 5). In addition, callus transformed with the NPT gene grew more rapidly than did control callus even when kanamycin was not used in the media. This result is perhaps attributable to preconditioning of the putative transgenic

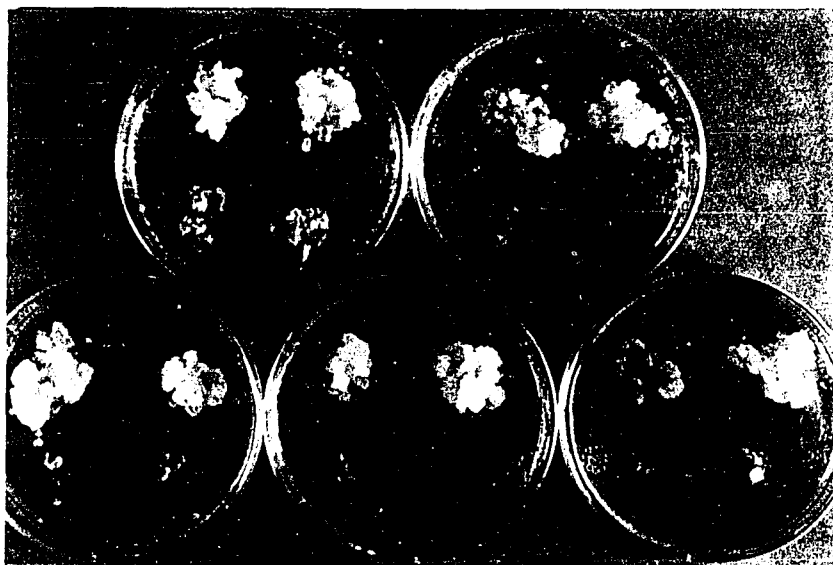


Figure 2. Growth comparison of transformed kanamycin-resistant calli and untransformed control calli of *P. alba* X *P. grandidentata* Sherrill clone after 4 weeks of culture on regeneration media containing 0, 50, 100, 150, and 200 mg/l kanamycin sulfate. The transformed kanamycin-resistant calli continued to grow at all test levels of kanamycin. The non-co-cultivated control calli were severely inhibited by 50 mg/l kanamycin sulfate

Table 5. Responses of transformed and non-transformed callus growth on the regeneration media containing various kanamycin concentrations. Callus growth was scored as percentage weight increase after three weeks of culture

Kanamycin conc. on regeneration medium	Transformed callus ( X 100%)	Non-transformed callus ( X 100%)
0 mg/l	3.02 $\pm$ 1.77	1.37 $\pm$ 0.64
50 mg/l	2.38 $\pm$ 0.67	0.31 $\pm$ 0.07
100 mg/l	3.03 $\pm$ 0.55	0.39 $\pm$ 0.46
150 mg/l	3.34 $\pm$ 0.28	0.28 $\pm$ 0.24
200 mg/l	2.98 $\pm$ 0.68	0.31 $\pm$ 0.06

calli on a high level cefotaxime medium designed to prevent *Agrobacterium* growth during previous subcultures. Mathias and Boyd (1986) have reported that cefotaxime stimulates callus growth of bread wheat (*Triticum aestivum* L EM. Thell). These phenotypic results indicate that the neomycin phosphotransferase gene is expressed in the callus tissue of the Sherrill clone. Similar results have been reported earlier in which various herbaceous plants such as tobacco, tomato, potato, *Arabidopsis* (An et al., 1986), white clover (White and Greenwood, 1987), *Stylosanthes* sp. (Manners, 1987) and *Medicago varia* (Deak et al., 1986), were successfully transformed with a neomycin phosphotransferase gene using binary vector pGA472.

At present, success to regenerate shoots from transformed calli remain limited. In the future, confirmation of gene transfer and whole plant regeneration from transformed calli will be conducted using an NPT assay (Reiss et al., 1984), Southern hybridization (Southern, 1975) and various regeneration techniques.

Toward the establishment of an efficient transformation and regeneration system of *Populus alba* X *P. grandidentata*, this preliminary study demonstrated the following: 1) *Agrobacterium* strain A281 containing pTiBo542 appears to be well suited to serve as a helper plasmid for binary vector systems; 2) A relatively low kanamycin sulfate concentration (10 mg/l) inhibited adventitious shoot initiation from leaf discs; and 3) pGA472 is effective for transferring NPT II gene into woody species as well as various herbaceous species.

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## OVERALL SUMMARY AND DISCUSSION

Six aspects involved in biotechnology applications of *Populus alba* X *P. grandidentata* micropropagation were studied: 1) the influences of medium consistency and shoot density on *in vitro* shoot proliferation, 2) protoplast isolation and culture of *in vitro* cultured leaf explants, 3) morphogenetic potential of *in vitro* cultured leaf, internode, and root explants, 4) influences of subculturing period and different culture media on cold storage of the plantlets, 5) *in vivo* and *in vitro* polyploidy induction, and 6) transformation by an *Agrobacterium* binary vector.

The *in vitro* shoot proliferation of this hybrid polar was affected by the medium consistency and shoot density, but not by different sizes of vessels that were tested. After 4 weeks of culture, the fresh weight and number of shoots per explant on liquid medium were significantly greater than those on agar-solidified medium. There was little difference in the number of shoots produced between vessels with 1 or 2 shoots per vessel, but there were fewer shoots produced when 3 shoots were placed in each vessel.

Protoplast-source material and enzyme strength had a significant influence on yield of protoplasts. The yield of protoplasts from *in vitro* cultured 1 month-old plantlets was more than that from greenhouse grown 4 month-old stock plants. Protoplast-derived hybrid poplar cells survived over 3 weeks in culture and some continuous cell divisions were evident.

Morphogenetic responses of the *in vitro* cultured explants depend upon the explant source and the combination of exogenously applied plant growth regulators. Leaf explants formed the most shoots, roots, and calli, while

root explants formed the least. Abaxial side culture of entire leaf explants was best suited for inducing adventitious shoots.

Subculturing period preceding cold storage, plantlet condition, and culturing medium all had important influences on survival at 4°C in darkness. Plantlets possessing 4-6 axillary shoots that were subcultured on shoot proliferation medium for one month preceding cold storage could be stored at 4°C air temperature in darkness for 24 months and still recover suitable multiplication potential.

The Crandon clone of the hybrid poplar was treated with various colchicine concentrations and treatment durations to determine the best method for inducing polyploidy through *in vivo* and *in vitro* treatment of axillary buds. Chimeric shoots of mixed 2n-4n ploidy level were produced from *in vivo* treatment with 24 h. duration with 1% concentration and 48 h. duration with 2% concentration, but not from *in vitro* treatments. The adventitious shoots produced from chimera leaf discs through *in vitro* culture exhibited either normal or colchipoide appearance.

Of the two *Agrobacterium* strains, A281 strain containing plasmid pTiBo542 appears to be well suited to serve as a helper plasmid for binary vector systems. A relatively low kanamycin concentration (10 mg/l) inhibited adventitious shoot initiation from leaf discs on regeneration medium. Transformed kanamycin resistant calli were obtained by culturing *Agrobacterium* binary vector inoculated leaf discs on selective regeneration medium. The transformed kanamycin resistant calli continued to grow on the regeneration medium containing kanamycin sulfate levels up to 200 mg/l.

Further suggestions for additional research include:



1) To reduce the vitrification symptoms of axillary shoot proliferation in liquid medium, various cellulose rods (Sorbarod System, from Baumgartner Papiers Co., Switzerland) have great potential. Our preliminary results indicated that axillary shoots which were cultured on cellulose rods in liquid medium produced as many multiple shoots as in liquid medium alone but without vitrification. This simple technique could allow for automatic shoot hedge production within a single culture vessel. Through weekly or monthly liquid nutrient replenishment, shoots could be periodically harvested without the need to transfer the cultures.

2) More intensive studies including stress-free culture environments (e.g. eliminating ammonium, agar, and light) are required to develop a generalized regeneration technique for protoplast culture.

3) Applications of physical treatments (e.g. puncture of the leaf surface) and various plant growth regulator substitutions (e.g. 2,4-D vs. NAA) in explant culture medium need to be tested to determine their effect on somatic embryogenesis from *Populus* species. The optimal culture conditions for explant source material need to be defined in order to optimize organogenesis or somatic embryogenesis.

4) To successfully preserve germplasm through cold storage of *in vitro* cultured plantlets, factors other than subculturing period preceding cold storage and different culture media need to be tested. Additional factors worthy of consideration include: storage temperature, nutrient composition of the culture medium, photoperiod, and light intensity.

5) Identification and histological classification of ploidy levels in chimeric leaves is needed. This will require more adventitious shoot

induction from *in vitro* chimeric leaf explants. In addition, *in vitro* treatments of axillary buds using higher concentrations of colchicine (> 0.1%) are required.

6) To obtain the maximum number of transformed shoots from leaf disc co-cultivation with *Agrobacterium*, leaf age, explant culture conditions, amount of *Agrobacterium*, duration of co-cultivation, and regeneration method need to be assessed.

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